

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/62, C07K 19/00, A61K 38/36, 38/57, C12N 1/21 // C07K 14/745, 14/81</b>		<b>A1</b>	(11) International Publication Number: <b>WO 97/20939</b>
			(43) International Publication Date: 12 June 1997 (12.06.97)
(21) International Application Number: PCT/US96/18756 (22) International Filing Date: 22 November 1996 (22.11.96) (30) Priority Data: 08/566,459 1 December 1995 (01.12.95) US 08/566,800 4 December 1995 (04.12.95) US (71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US). (72) Inventors: KELLEY, Robert, F.; 1029 San Felipe Avenue, San Bruno, CA 94066 (US). LAZARUS, Robert, A.; 237 Hillcrest Boulevard, Millbrae, CA 94030 (US). LEE, Geoffrey, F.; 1170 Mason Drive, Pacifica, CA 94044 (US). (74) Agents: KUBINEC, Jeffrey, S. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: TISSUE FACTOR - KUNITZ DOMAIN FUSION PROTEINS AS FACTOR VIIa INHIBITORS			
(57) Abstract  The invention provides compositions having a Factor VIIa active site inhibitor domain and a tissue factor domain for the inhibition of FVIIa. The invention also provides pharmaceutical compositions comprising the novel compositions as well as their use in diagnostic, therapeutic, and prophylactic methods.			

BEST AVAILABLE COPY

found to be important for FVIIa binding on the basis of mutagenesis results (Kelley et al., (1995) *supra*). A set of residues, Trp158, Lys159, Ser163, Gly164, Lys165, Lys166, and Tyr185 play a role in the coagulant function of soluble TF but do not participate in FVIIa binding (Kelley et al., (1995) *supra*).

TF is expressed constitutively on cells separated from plasma by the vascular endothelium (Carson, S. D. and J. P. Brozna, (1993) *Blood Coag. Fibrinol.* 4:281-292). Its expression on endothelial cells and monocytes is induced by exposure to inflammatory cytokines or bacterial lipopolysaccharides (Drake et al., (1989) *J. Cell Biol.* 109:389). Upon tissue injury, the exposed extracellular domain of TF forms a high affinity, calcium dependent complex with FVII. Once bound to TF, FVII can be activated by peptide bond cleavage to yield serine protease FVIIa. The enzyme that catalyzes this step *in vivo* has not been elucidated, but *in vitro* FXa, thrombin. TF-FVIIa and FIXa can catalyze this cleavage (Davie, et al., (1991) 30:10363-10370). FVIIa has only weak activity upon its physiological substrates FX and FIX whereas the TF-FVIIa complex rapidly activates FX and FIX.

The TF-FVIIa complex constitutes the primary initiator of the extrinsic pathway of blood coagulation (Carson, S. D. and Brozna, J. P., (1993) *Blood Coag. Fibrinol.* 4:281-292; Davie, E. W. et al., (1991) *Biochemistry* 30:10363-10370; Rapaport, S. I. and L. V. M. Rao, (1992) *Arterioscler. Thromb.* 12:1111-1121). The complex initiates the extrinsic pathway by activation of FX to Factor Xa (FXa), FIX to Factor IXa (FIXa), and additional FVII to FVIIa. The action of TF-FVIIa leads ultimately to the conversion of prothrombin to thrombin, which carries out many biological functions (Badimon, L. et al., (1991) *Trends Cardiovasc. Med.* 1:261-267). Among the most important functions of thrombin is the conversion of fibrinogen to fibrin, which polymerizes to form a clot. The TF-FVIIa complex also participates as a secondary factor in extending the physiological effects of the contact activation system.

The involvement of this plasma protease system has been suggested to play a significant role in a variety of clinical manifestations including arterial and venous thrombosis, septic shock, adult respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC) and various other disease states (Haskel, E. J. et al., (1991) *Circulation* 84:821-827); Holst, J. et al., (1993) *Haemostasis* 23 (suppl. 1):112-117; Creasey, A. A. et al., (1993) *J. Clin. Invest.* 91:2850-2860; see also, Colman R. W. (1989) *N. Engl. J. Med* 320:1207-1209; Bone, R. C. (1992) *Arch. Intern. Med.* 152:1381-1389). Overexpression and/or aberrant utilization of TF has been linked to the pathophysiology of both thrombosis and sepsis (Taylor et al., (1991) *Circ. Shock* 33:127; Warr et al., (1990), *Blood* 75:1481; Pawashe et al., (1994) *Circ. Res.* 74:56). TF is expressed on cells found in the atherosclerotic plaque (Wilcox et al., (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:2839). Additionally, TF has been implicated in tumor metastasis (Bromberg et al., (1995) *Proc. Natl. Acad. Sci., USA*, 92:8205).

The presence in serum of an endogenous inhibitor of TF-FVIIa has long been recognized (Schneider, C. L., (1946) *Am. J. Physiol.* 149:123). Following initiation of the coagulation cascade, TF-FVIIa is regulated by tissue factor pathway inhibitor (TFPI), a feedback inhibitor that prevents further activation of zymogen substrates (Broze Jr., G. J. et al., (1990) *Biochemistry* 29:7539-7546; Broze Jr., G. J., (1992) *Semin. Hematol.* 29:159-169). TFPI is also known as LACI or EPI for lipoprotein associated coagulation inhibitor and extrinsic pathway inhibitor, respectively. TFPI contains an acidic amino terminal region followed by three Kunitz-type domains and a basic carboxyl terminal region. TFPI is thought to inhibit TF-FVIIa in a FXa dependent manner, first binding FXa via the second Kunitz domain followed by binding FVIIa via the first Kunitz domain (Girard,

## TISSUE FACTOR - KUNITZ DOMAIN FUSION PROTEINS AS FACTOR VIIa INHIBITORS

## Field of the Invention

This invention relates to novel compositions which bind to and inhibit an activity associated with plasma Factor VII/Factor VIIa. Specifically, the invention provides molecules having a Factor VIIa active site inhibitor domain and a tissue factor domain. According to a preferred aspect, the two domains of the hybrid molecule of the present invention are linked via flexible peptide linker domain. The invention also relates to pharmaceutical compositions comprising the novel compositions as well as their use in diagnostic, therapeutic, and prophylactic methods.

## Description of Related Disclosures

Factor VIIa (FVIIa) is a two-chain, 50 kilodalton (kDa), vitamin K-dependent, plasma serine protease which participates in the complex regulation of *in vivo* hemostasis. Factor VIIa is generated by proteolysis of a single peptide bond from its single chain zymogen, Factor VII, which is present at approximately 0.5 µg/ml in plasma. The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal peptide bond. In human Factor VII, the cleavage site is at Arg152-Ile153 (Hagen et al., (1986) Proc. Natl. Acad. Sci. USA 83:2412-2416; Thim et al., (1988) Biochem. 27:7785-7793,). In the presence of calcium ions, Factor VIIa binds with high affinity to tissue factor (TF), an integral membrane protein. TF is a cofactor for Factor VIIa, enhancing the proteolytic activation of its substrates Factors VII (FVII), Factor IX (FIX) and Factor X (FX).

TF is a 263 amino acid residue glycoprotein composed of a 219 residue extracellular domain, a single transmembrane domain, and a short cytoplasmic domain (Morrissey, J.H., et al., (1987) 50:129). The TF extracellular domain is composed of two fibronectin type III domains of about 105 amino acids each. The binding of FVIIa is mediated entirely by the TF extracellular domain (Muller et al., (1994) Biochem. 33:10864-10870; Gibbs et al., (1994) Biochem. 33:14003-14010; Ruf et al., (1994) Biochem. 33:1565-1572). The structure of the TF extracellular domain has recently been determined by x-ray crystallography (Harlos et al., (1994) Nature 370:662-666; Muller et al., (1994) Biochemistry 33:10864).

The TF extracellular domain has also been extensively characterized by alanine scanning mutagenesis (Kelley et al., (1995) Biochemistry, 34:10383-10392; Gibbs et al., (1994) *supra*; Ruf et al., (1994) *supra*). Residues in the area of amino acids 16-26 and 129-147 contribute to the binding of FVIIa as well as the coagulant function of the molecule. Residues Lys20, Trp45, Asp58, Tyr94, and Phe140 make a large contribution (1 kcal/mol) to the free energy ( $\Delta G$ ) of binding to FVIIa (Kelley et al., (1995) *supra*).

Residues in the area of amino acids 157-168 contribute to the procoagulant function of TF-FVIIa (Kelley et al., (1995) *supra*; Ruf et al., (1992) J. Biol. Chem. 267:22206-22210) but are not important for FVII/FVIIa binding. It has been shown that lysine residues 165 and 166 are important to TF cofactor function but do not participate in FVIIa complex formation (Roy et al., (1991) J. Biol. Chem. 266:22063; Ruf et al., (1992) J. Biol. Chem. 267:6375). Alanine substitution of these lysine residues results in a decreased rate of FX activation catalyzed by the TF-FVIIa complex (Ruf et al., (1992) *supra*). Lysine residues 165 and 166 are located on the C-terminal fibronectin type III domain of TF on the opposite surface of the molecule from residues

the scissile peptide bond of the substrate or inhibitor and fits into the  $S_1$  binding site as defined by Schechter and Berger (1967) *Biochem. Biophys. Res. Commun.*, 27:157-162. Cleavage between the  $P_1$  and  $P_1'$  residues occurs very slowly if at all (Bode, W. and Huber, R., (1992) *Eur. J. Biochem.* 204:433-451; Laskowski, M., Jr. and Kato, I., (1980) *Annu. Rev. Biochem.* 49:593-626).

5 Of the many interactions between the serine protease subsites and the side chains in the primary binding loop of Kunitz-type domain serine protease inhibitors ( $P_5-P_4'$ ) (Bode, W. and Huber, R., (1992) *Eur. J. Biochem.* 204:433-451; Laskowski, M., Jr. and Kato, I., (1980) *Annu. Rev. Biochem.* 49:593-626), the interactions of the  $P_1$  residue with the specificity pocket are energetically most important and therefore represent the primary specificity determinants. Although the amino acid at the  $P_1$  position generally dominates the affinity of inhibitors  
10 for the serine protease active site (Scott, C. F. et al., (1987) *Blood* 69:1431-1436; Laskowski, M., Jr. and Kato, I., (1980) *supra*; Beckmann, J. et al., (1988) *Eur. J. Biochem.* 176:675-682; Sinha, S. et al., (1991) *J. Biol. Chem.* 266:21011-21013), residues outside this region (11-14 and 16-19 as well as residue 34 of secondary binding loop) are also known to play a role in binding affinity and specificity towards serine proteases (Kossiakoff, A. A. et al., (1993) *Biochem. Soc. Trans.* 21:614-618); Roberts, B. L. et al., (1992) *Proc Natl Acad Sci USA* 89:  
15 2429-2433). The crystal structures of Kunitz-type domains reveal residues within the primary binding loop that are likely to make contact with the serine protease (Hynes, T. R. et al., (1990) *supra*; Bode, W. and Huber, R., (1992) *supra*; Kossiakoff, A. A. (1993), *supra*).

Antagonists to tissue factor have also been demonstrated. Neutralizing anti-TF monoclonal antibodies have been shown to prevent death in a baboon model of sepsis (Taylor et al., (1991) *Circ. Shock* 33:127),  
20 attenuate endotoxin-induced DIC in rabbits (Warr et al., (1990), *Blood* 75:1481), and to prevent thrombus reformation in a rabbit model of arterial thrombosis (Pawashe et al., (1994) *Circ. Res.* 74:56). Incorporation of lysine to alanine mutations at amino acid residues 165 and 166 of soluble tissue factor results in a molecule that binds FVIIa with an affinity comparable to wild type soluble tissue factor (Kelley et al., (1995) *supra*). TF residues Lys 165 and Lys 166 have been shown to be important for inhibition of TF-FVIIa by the TFPI-FXa  
25 complex (Rao and Ruf, (1995) *Biochemistry* 34:10867-10871). The rate of inhibition by TFPI-FXa is decreased for FVIIa in complex with a TF mutant containing Lys to Ala substitutions at positions 165 and 166 as compared to FVIIa in complex with wild-type TF. Variants of tissue factor have also been produced that bind to functional FVIIa but which have decreased procoagulant activity (International Publication No WO 94/28017). These molecules inactivate the cofactor activity of tissue factor in the TF-FVIIa complex but do not alter the ability of  
30 the molecule to complex FVIIa.

### Summary of the Invention

The present invention provides compositions which inhibit a TF-FVIIa mediated or associated process such as the catalytic conversion of FVII to FVIIa, FIX to FIXa, or FX to FXa and thereby block initial events  
35 of the extrinsic pathway of blood coagulation. In addition, the compositions of the present invention are capable of neutralizing the thrombotic effects of endogenous tissue factor by competing with endogenous tissue factor for binding to FVII or FVIIa. Advantageously, the compositions allow for a potent inhibition of the TF-FVIIa complex providing, in preferred embodiments, for low dose pharmaceutical formulations. The compositions of

T. J. (1989) et al., Nature 338:518-520). In the absence of FXa, TFPI is a poor inhibitor of the TF-FVIIa complex (Girard, T. J. (1990) et al., Science 248:1421-1424).

5 Variants of TFPI have been made that also inhibit TF-FVIIa activity. A variant that contains the first two Kunitz domains (residues 1-161) has been made and characterized (Hamamoto et al. (1993) J. Biol. Chem. 268:8704-8710; Petersen et al. (1993) J. Biol. Chem. 268:13344-13351). International Publication No. WO 91/02753 as well as U.S. Patent 5,312,736 describe TFPI variants that retain their serine protease inhibitory activity but have a decreased binding affinity for heparin. A TFPI-FXa hybrid protein is able to inhibit TF-FVIIa induced coagulation of normal plasma in an *in vitro* coagulation assay with a greater activity than TFPI alone (Girard et al., (1990) Nature 248:1421-1424).

10 TFPI and variants have been shown to affect hemostasis in animal models of arterial reocclusion after thrombolysis (Haskel, E. J. et al., (1991) Circulation 84:821-827), venous thrombosis (Holst, J. et al., (1993) Haemostasis 23 (suppl. 1):112-117), and disseminated intravascular coagulation resulting from septic shock (Creasey, A. A. et al., (1993) J. Clin. Invest. 91:2850-2860). However, TFPI may not have all of the properties desired for an anticoagulant agent for the treatment of thrombotic disease.

15 Recently, the serpin antithrombin III (ATIII) has also been shown to inhibit TF-FVIIa activity in the presence of heparin (Rao, L. V. M. et al., (1993) Blood 81:2600-2607; Lawson, J. H. (1993) et al., J. Biol. Chem. 268:767-770; Broze Jr., G. J. et al., (1993) Blood 82:1679-1680; Mann, K. G., (1993) Blood 82:1680-1681). Inhibition of TF-FVIIa by TFPI is reversible, whereas inhibition by ATIII is essentially irreversible. The relative importance of ATIII/heparin inhibition of TF-FVIIa versus TFPI *in vivo* is unknown.

20 Bovine pancreatic trypsin inhibitor (BPTI), also referred to as aprotinin, has recently been shown to competitively inhibit TF-FVIIa activity, albeit with relatively weak affinity ( $K_i \approx 30 \mu\text{M}$ ) (Chabbat, J. et al., (1993) Thromb Res 71:205-215). In addition, BPTI has recently been shown to inhibit TF-induced coagulation; however, ca. 75  $\mu\text{M}$  was needed to prolong the clotting time 1.4-fold in a PT assay (van den Besselaar, A. M. H. P. et al., (1993) Thromb. Haemostas. 69:298-299).

25 Recently, the Kunitz domain of Alzheimer's amyloid  $\beta$ -protein precursor (APPI), which is structurally similar to BPTI (Hynes, T., et al., (1990) Biochemistry 29:10018-10022), was used as a scaffold for phage display of a large library of variants to select potent and specific active site inhibitors of tissue factor-Factor VIIa (Dennis, M.S., and Lazarus, R.A., (1994) J. Biol. Chem. 269:22129-22136; Dennis, M.S., and Lazarus, R.A., (1994) J. Biol. Chem. 269:22137-22144). International Publication No. WO 95/23860 describes the generation  
30 of Kunitz-type inhibitors of Factor VIIa.

The foregoing FVIIa inhibitors have been classified as Kunitz-type serine protease inhibitors (TFPI, BPTI, APPI, for example). Kunitz-type serine protease inhibitors are a well characterized family of proteins that exhibit extensive structural homology including a characteristic tertiary fold containing an extended binding loop that fits into the active site of the cognate serine protease (Bode, W., and Huber, R., (1992) Eur. J. Biochem. 204:433-451). Kunitz-type serine protease inhibitors are known to be slow, tight-binding, reversible inhibitors  
35 of serine proteases such as FVIIa that bind to the active site and inhibit according to the standard mechanism (Laskowski, Jr., M. and Kato, I., (1980) Ann. Rev. Biochem. 49:593-626).

In a Kunitz-type domain/serine protease complex, the side chain of residue 15 of the Kunitz-type domain fills the  $P_1$  position preceding the scissile peptide bond. The  $P_1$  residue refers to the position preceding

### Brief Description of the Drawings

Figure 1 depicts a schematic outline of selected enzymes and mediators that modulate the coagulation, contact, fibrinolytic, inflammatory, and complement pathways. Activation of these pathways can lead to the clinical states indicated.

5 Figure 2 depicts a model of an exemplary composition. The composition comprises a fusion protein having a TF domain, an active site inhibitor domain (APPI), and a linker domain. The structure of TF determined at 2.4 Å resolution (Muller et al., (1994) *supra*) was used for the TF portion of the molecule. The Kunitz-type domain is based upon the structure determined for APPI (Hynes et al., (1990) *supra*). A 22 residue polypeptide linker was modeled in extended conformation to connect the C-terminus of the Kunitz-domain to the N-terminus of TF. The distance between these termini is about 60 Å. The C-terminus of TF is at the bottom  
10 of the molecule.

Figure 3 depicts a schematic diagram of the construction of the fusion gene for the bacterial expression of a representative hybrid protein composition. Only the fusion gene and promotor regions of the plasmid are shown. The fusion genes were constructed by ligating the Pst/BamHI fragment encoding the TF7I-C Kunitz-type domain with pZTFAA and oligonucleotides prepared by chemical synthesis.  
15

Figure 4 depicts a rendering of a representative polyacrylamide gel showing samples obtained from the various purification steps for a representative hybrid molecule including freeze-thawing, then osmotically shocking, cells to obtain a periplasmic shockate (lane 1), immunoaffinity chromatography using D3 monoclonal anti-tissue factor antibody to isolate TF-containing proteins (lane 2), followed by size exclusion chromatography to remove high molecular weight species (lane 3).  
20

Figure 5 depicts the effect of fusion proteins with 1 to 6 linker modules (KDTF1-KDTF6) on TF-FVIIa-dependant FX activation. Fusion protein inhibition of TF-FVIIa interactions was quantified in a FX activation assay. The assay was performed by incubating 100 pM FVIIa with 1 nM inhibitor in TBS, pH 7.5, containing 5 mM  $\text{CaCl}_2$  for 1 hr, then adding 1/20th volume of a solution containing 10nM membrane-bound TF. After  
25 further incubation for 1 hr, FX was added to 190 nM. At appropriate times, aliquots were removed and the reaction quenched with an equal volume of 50 mM EDTA. Activated FXa in quenched samples was then quantified using SPECTROZYME™-FXa as chromogenic substrate.

Figure 6 depicts the inhibition of TF-FVIIa by a representative hybrid molecule KDTF5 (•), a soluble K165A, K166A TF mutant (hTFAA) (■), and TF7I-C, a Kunitz-type serine protease inhibitor (●) for TF-FVIIa.  
30 Values were determined in a modified FX activation assay, performed with 2-fold more concentrated FVIIa and membrane-TF, and with varying inhibitor concentrations.  $K_i^*$  determinations were made by fitting the fractional rate vs. inhibitor concentration data to equation 1 (*infra*).

Figure 7 depicts the effects of TF-FVIIa inhibitors on the clotting time determined in a prothrombin (PT) time assay. Coagulation was initiated by mixing citrated human plasma containing inhibitor with an equal  
35 volume of a solution containing membrane-bound TF (mTF) and  $\text{CaCl}_2$ . Fold-prolongation of clotting time was defined as the clotting time in the presence of inhibitor divided by the clotting time in its absence (PBS control). This latter time was  $53.2 \pm 2.7$  seconds. Data are shown for KDTF5 (•), TF7I-C (•), hTFAA (■), and an equimolar mixture of TF7I-C and hTFAA (•).

the present invention are useful in therapeutic and prophylactic methods for inhibiting TF-FVIIa mediated or associated processes.

The compositions of the present invention comprise a FVIIa active site inhibitor domain and a TF domain. According to a preferred aspect of the present invention, the TF domain competes with endogenous TF  
5 for binding to FVII/FVIIa but has a decreased ability to act as a cofactor for the conversion of FVII to FVIIa, FDX to FDXa or FX to FXa. The FVIIa active site inhibitor domain of the compositions of the present invention binds to the serine protease active site of FVIIa and prevents catalytic activation of its zymogen substrates. In a preferred embodiment the active site inhibitor domain of the composition of the present invention is a reversible active site serine protease inhibitor molecule.

10 According to certain preferred aspects of the present invention the compositions further comprise a linker domain located between the FVIIa active site inhibitor domain and the TF domain. The linker domain is preferably a hydrophilic flexible domain comprising a  $-(\text{Gly})_4\text{-Ser-}$  linker module.

In one embodiment, the composition of the present invention is a polypeptide and the invention encompasses a composition of matter comprising an isolated nucleic acid, preferably DNA, encoding the  
15 polypeptide of the invention. According to this aspect, the invention further comprises an expression control sequence operably linked to the DNA molecule, an expression vector, preferably a plasmid, comprising the DNA molecule, where the control sequence is recognized by a host cell transformed with the vector, and a host cell transformed with the vector.

The composition of the present invention may be made by a process which includes the steps of  
20 isolating or synthesizing nucleic acid sequences encoding any of the amino acid sequences of the invention, ligating the nucleic acid sequence into a suitable expression vector capable of expressing the nucleic acid sequence in a suitable host, transforming the host with the expression vector into which the nucleic acid sequence has been ligated, and culturing the host under conditions suitable for expression of the nucleic acid sequence, whereby the protein encoded by the selected nucleic acid sequence is expressed by the host. Preferably, the  
25 polypeptide is then recovered from the host cell culture. In this process, the ligating step may further contemplate ligating the nucleic acid into a suitable expression vector such that the nucleic acid is operably linked to a suitable secretory signal, whereby the amino acid sequence is secreted by the host. The secretory signal may be selected from the group consisting of the leader sequence, for example, of stII, lamB, herpes gD, lpp, alkaline phosphatase, invertase, and alpha factor and is preferably stII.

30 The present invention further extends to therapeutic applications for the compositions described herein. Thus the invention includes a pharmaceutical composition comprising a pharmaceutically acceptable excipient and the composition of the invention. Pharmaceutical compositions comprising these molecules can be used in the treatment or prophylaxis of thrombotic or coagulopathic related diseases or disorders including vascular diseases and inflammatory responses. These applications include, for example, a method of treating a mammal  
35 for which inhibiting TF-FVIIa is indicated comprising administering a pharmaceutically effective amount of the pharmaceutical composition to the mammal. Such indications include; deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplasty (PTCA), stroke, tumor metastasis, inflammation, septic shock, hypotension, ARDS, and DIC. The compositions of the present invention may also be used as an adjunct in thrombolytic therapy.

syndrome (ARDS), disseminated intravascular coagulopathy (DIC) and other diseases. The TF-FVIIa related disorder is not limited to in vivo coagulopathic disorders such as those named above but includes ex vivo TF-FVIIa related processes such as coagulation that may result from the extracorporeal circulation of blood, including blood removed in-line from a patient in such processes as dialysis procedures, blood filtration, or blood bypass during surgery.

The terms "tissue factor protein" and "wild type tissue factor" are used to refer to a polypeptide having an amino acid sequence corresponding to a naturally occurring mammalian tissue factor or a recombinant tissue factor having an amino acid sequence of a naturally occurring tissue factor which is capable of inducing blood coagulation through its interaction with plasma FVII/FVIIa. Naturally occurring TF includes human species as well as other animal species such as rabbit, rat, porcine, non human primate, equine, murine, and ovine tissue factor. The amino acid sequence of the mammalian tissue factor proteins are generally known or obtainable through conventional techniques. The human sequence as well as the number given to the amino acids are those described by Morrissey, J.H., et al., (1987) 50:129.

"hTFAA" and "K165A:K166AsTF" refer to a soluble tissue factor protein variant having the amino acid sequence of human TF as described above from amino acid 1 to amino acid 219 and wherein the naturally occurring Lys amino acids at positions 165 and 166 are replaced with Ala residues.

By "substitution" of any amino acid is meant that an amino acid of the wild-type tissue factor has been replaced by chemical or enzymatic or other appropriate means with a moiety other than a wild-type amino acid.

The terms "Kunitz-type serine protease inhibitor domain," "Kunitz-type domain," "Kunitz domain," "KD" and the like are used interchangeably herein to refer to an approximately 58 amino acid residue polypeptide characterized by a conservation of cysteine residues with the serine protease inhibitor BPTI (bovine pancreatic trypsin inhibitor; Creighton and Charles, (1987) Cold Spring Harbor Symp. Quant. Biol. 52:511-519) first isolated in crystalline form in 1936 (Kunitz, M., and Northop, J.H., (1936) J. Gen. Physiol. 19:991-1007). Kunitz domains share cysteine residue placement, tertiary folding, and structural characteristics. A family of proteins has been identified containing one, two or three Kunitz domains. The family includes: LACI (lipoprotein-associated coagulation inhibitor, also TFPI; Broze, Jr. G. J., et al., (1990) Biochemistry 29:7539-7546); APPI (Alzheimer's amyloid  $\beta$ -protein precursor; Hynes, T.R., (1990) Biochemistry 29:10018-10022); the  $\alpha$ -3 chain of human type VI collagen (see WO 93/14119) and inter- $\alpha$ -trypsin inhibitor (Hochstrasser, K., E., (1985) Biol. Chem. Hoppe-Seyler 366:473).

Kunitz domains contain six specifically spaced cysteines that are present naturally in disulfide bonds (Bode, W., and Huber, R., (1992) Eur. J. Biochem. 204:433-451). The three disulfide bridges stabilize the protein and are partially responsible for the overall 3-dimensional folding characteristic of a Kunitz domain. In the 58 residue Kunitz type serine protease BPTI and APPI, cysteines are present at residues 5, 14, 30, 38, 51, and 55. The removal of a one disulfide bridge, however is not accompanied by a large structural change (Eigenbrot, C., et al., (1990) Protein Eng. 3:591-598).

The crystal structure of Kunitz domain type serine protease inhibitors has been determined for BPTI and APPI (Hynes, T.R., et al., (1990) Biochem., 29:10018-10022). A central anti-parallel three-stranded  $\beta$ -sheet and a C-terminal  $\alpha$ -helix form the core of the domain (Bode, W., and Huber, R., *supra*). The segments of the core domain form the supporting scaffold for the exposed primary binding loop of the properly folded protein



Figure 8 depicts the effects of TF-FVIIa inhibitors on the clotting time determined in an activated partial thromboplastin time (APTT) assay. The sample (citrate human plasma and inhibitor) and activator (Instrumentation Laboratories Cephalin + Activator APTT Reagent) were incubated together for 70 seconds at 37 °C, then  $\text{CaCl}_2$  was added and the clotting time was determined. Fold prolongation of clotting time was defined as the clotting time in the presence of the indicated concentration of inhibitor divided by the clotting time in the absence of inhibitor. This latter time was  $63.7 \pm 9.0$  seconds. Data are shown for KDTF5 (•), TF7I-C (•), hTFAA (■), and equimolar mixture of TF7I-C and hTFAA (▲).

### Detailed Description of the Preferred Embodiments

#### Definitions

Abbreviations used throughout the description include: FIXa for Factor IXa; FXIa for Factor XIa; FXa for Factor Xa; TF for tissue factor; FVII for zymogen factor VII; FVIIa for Factor VIIa; TF-FVIIa for tissue factor-Factor VIIa complex; FVII/FVIIa for FVII and/or FVIIa; BPTI for basic pancreatic trypsin inhibitor; APPI for the Kunitz protease inhibitor domain of Alzheimer's amyloid  $\beta$ - protein precursor inhibitor (Hynes et al., (1990) *supra*); -(Gly)<sub>4</sub>-Ser-, and G<sub>4</sub>S for a 5 amino acid polypeptide having the amino acid sequence Gly-Gly-Gly-Gly-Ser; TFPI for tissue factor pathway inhibitor;  $K_i^*$  for apparent equilibrium dissociation constant; PT for prothrombin time; APTT for activated partial thromboplastin time.

A TF-FVIIa mediated or associated process or event, or equivalently, an activity associated with plasma FVIIa, according to the present invention is any event which requires the presence of TF-FVIIa. The general mechanism of blood clot formation is reviewed by Ganong, in Review of Medical Physiology, 13th ed., Lange, Lost Altos CA, pp411-414 (1987). Coagulation requires the confluence of two processes, the production of thrombin which induces platelet aggregation and the formation of fibrin which renders the platelet plug stable. As outlined in Figure 1, the process comprises several stages each requiring the presence of discrete proenzymes and procofactors. The process ends in fibrin crosslinking and thrombus formation. Fibrinogen is converted to fibrin by the action of thrombin. Thrombin, in turn, is formed by the proteolytic cleavage of prothrombin. This proteolysis is effected by FXa which binds to the surface of activated platelets and in the presence of FVa and calcium, cleaves prothrombin. TF-FVIIa is required for the proteolytic activation of FX by the extrinsic pathway of coagulation. Therefore, a process mediated by or associated with TF-FVIIa, or an activity associated with FVIIa includes any step in the coagulation cascade from the formation of the TF-FVII complex to the formation of a fibrin platelet clot and which initially requires the presence TF-FVIIa. For example, the TF-FVIIa complex initiates the extrinsic pathway by activation of FX to FXa, FIX to FIXa, and additional FVII to FVIIa. TF-FVIIa mediated or associated process, or FVIIa activity, can be conveniently measured employing standard assays such as those described in Roy, S., (1991) J. Biol. Chem. 266:4665-4668, and O'Brien, D., et al., (1988) J. Clin. Invest. 82:206-212 for the conversion of Factor X to Factor Xa in the presence of Factor VII and other necessary reagents.

A TF-FVIIa related disease or disorder is meant to include chronic thromboembolic diseases or disorders associated with fibrin formation including vascular disorders such as deep venous thrombosis, arterial thrombosis, stroke, tumor metastasis, thrombolysis, arteriosclerosis and restenosis following angioplasty, acute and chronic indications such as inflammation, septic shock, septicemia, hypotension, adult respiratory distress

and O'Brien, D., et al., (1988) J. Clin. Invest. 82:206-212) produces at least a 50% inhibition of the conversion of Factor X to Factor Xa in the presence of Factor VII and other necessary reagents. Preferably the tissue factor domain will produce at least a 50% inhibition at a 5-fold molar excess and more preferably at least a 50% inhibition at a 2-fold molar excess. In a more preferred embodiment the tissue factor domain of the present invention will produce at least a 50% inhibition of the conversion of Factor X to Factor Xa when present in a 1:1 stoichiometric ratio with wild type tissue factor protein. According to the present invention, TF domains include but are not limited to full length, phospholipid associated tissue factor domains having both a transmembrane domain and a cytoplasmic domain as well as TF domains wherein all or a portion of the transmembrane and/or cytoplasmic domain of wild type tissue factor have been deleted. Accordingly, in determining the biological activity of a TF domain of the present invention in a chromogenic assay as described, the wild-type tissue factor corresponds in type to the particular TF domain being tested. Therefore a full length TF domain comprising all or a portion of the transmembrane domain of wild-type tissue factor and associated with a phospholipid is tested in a biological assay with wild type TF of the corresponding type comprising all or a portion of the transmembrane domain in a similar phospholipid environment. Likewise, a soluble TF domain wherein the transmembrane and cytoplasmic domain have been deleted is assayed with a wild-type TF domain wherein the transmembrane and cytoplasmic domain have been deleted.

It is a characteristic of the TF domain of the present invention that the protein bind FVII/FVIIa. Accordingly, the TF domain of the present invention shares those residues with wild type TF protein that are necessary for the binding of TF to FVII/FVIIa. By "bind to FVII/FVIIa" is meant that the TF domain of the present invention has the ability to bind to FVII/FVIIa to a degree that TF domain can compete for binding with wild type TF at physiological concentrations. Preferred among the TF domains are those that have a KD for FVII/FVIIa of about between 10.0 picomolar (pM) and about 1 micromolar ( $\mu$ M) in a standard binding assay such as that described by Kelley et al., (1995) *supra*. More preferably the TF domain has a KD for FVII/FVIIa of about between 10 pM and 10 nanomolar (nM) and most preferably about between 10 pM and 1 nM.

The skilled artisan will recognize those residues in TF that contribute to the FVIIa binding (Kelley et al. (1995) *supra*; Gibbs et al., (1994) *supra*; Ruf et al., (1994) Biochemistry, 33, 1565-1572; Schullek et al., (1994) J. Biol. Chem. 269:19399-19403; Muller et al., (1994) 33:10864-10869). According to the present invention, the TF domain shares at least those residues with wild type TF which are required for FVIIa/FVII binding, as described. Preferably, the tissue factor domain will share at least about 80% sequence homology and more preferably between about 85%-95% sequence homology with wild-type tissue factor protein.

TF domains include but are not limited to full length, membrane bound or phospholipid associated tissue factor domains having both a transmembrane domain and a cytoplasmic domain. The TF domain also includes tissue factor domains wherein all or a portion of the transmembrane and/or cytoplasmic domain of wild type tissue factor have been deleted. Accordingly, the tissue factor domain of the molecule can be formulated as soluble protein having the carboxyl-terminal membrane anchor portion and cytoplasmic portion removed. Alternatively, a TF protein can be prepared as an integral membrane protein having a membrane anchor. The tissue factor domain having a complete membrane anchor domain is provided in association with phospholipid compositions such as liposome formulations.

(the "primary binding loop" as defined herein)(Bode, W., and Huber, R., *supra*). A secondary binding loop participates along with the primary binding loop to define the interface between the Kunitz domain and the cognate protease target (Rühlman, A., et al., (1973) J. Mol. Biol. 77:417-436).

The "primary binding loop" of a Kunitz domain is designated by  $P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'$  (residues 11-19 APPI). The term " $P_1$ " is used herein to refer to the position preceding the scissile peptide bond of the serine protease inhibitors as previously defined. Similarly, the term " $P_1'$ " is used to refer to the position following the scissile peptide bond of the inhibitor. Increasing numbers refer to the next consecutive position preceding (e.g.,  $P_2$  and  $P_3$ ) and following (e.g.,  $P_2'$  and  $P_3'$ ) the scissile bond. The residue numbering corresponds to that of BPTI such that residue 15 is at the  $P_1$  position.

The term "active site" and the like when used herein with reference to FVIIa refer to the catalytic and zymogen substrate binding site, including the " $S_1$ " site of FVIIa as that term is defined by Schechter, I., and Berger, A., (1967) Biochem. Biophys. Res. Commun. 27:157-162

The term "amino acid" within the scope of the present invention is meant to refer to naturally occurring L alpha amino acids or residues. The commonly used one and three letter abbreviations for amino acids are used herein (Lehninger, A.L., Biochemistry, 2d ed., pp. 71-92, (1975), Worth Publishers, New York).

#### Modes for Carrying out the Invention

The hybrid molecules of the present invention comprise at least two distinct domains. Each molecule of the present invention contains a Factor VIIa active site inhibitor domain which binds to the active site of FVII/FVIIa and a tissue factor domain which binds to FVII/FVIIa through protein-protein interactions at the cofactor binding site. According to the present invention the active site inhibitor-domain is linked to the N-terminus of the tissue factor domain, preferably via a flexible amino acid linker domain.

##### 1. Tissue Factor Domain

The tissue factor domain of the compositions of the present invention is a tissue factor protein variant which by virtue of, for example, an amino acid substitution, insertion, or deletion in the amino acid sequence of a mammalian tissue factor protein, or by virtue of glycosylation variation including deglycosylated or unglycosylated tissue factor molecules, is capable of neutralizing the ability of tissue factor to induce blood coagulation while retaining at least its ability to bind to Factor VII/VIIa. In a particular embodiment, the TF domain in complex with FVIIa has a decreased ability to catalyze the conversion of substrate Factor X to Factor Xa product. Thus, the tissue factor domain of the present invention is believed to, without limitation, compete with wild-type tissue factor for the cofactor binding site of Factor VII/VIIa, thereby neutralizing or preventing wild type tissue factor from acting as a cofactor in the coagulation cascade.

The tissue factor domain of the present invention is capable of inhibiting or neutralizing any available tissue factor protein from inducing blood coagulation through the extrinsic coagulation pathway (see, Bach, R., (1988) CRC Crit. Rev. Biochem. 23:339-368). As will be appreciated by the skilled artisan the term "neutralize" is a relative term. Thus the term "neutralize" when used to describe the biological activity of the tissue factor domain of the present invention means a tissue factor protein variant that when added in a 10-fold molar excess to wild-type tissue factor in a standard chromogenic assay (see, Roy, S., (1991) J. Biol. Chem. 266:4665-4668,

portion of wild type tissue factor, residues 244-263, removed, as well as the transmembrane region at residues 220-243. Any of the tissue factor variants may appropriately form the TF domain of the present invention. International Publication No. WO 94/28017 also describes TF variants that are able to bind FVII/FVIIa and have a reduced procoagulant cofactor activity. Most preferred among the molecules described therein are a tissue factor protein having an amino acid sequence homologous to a wild type tissue factor protein and wherein at least one amino acid associated with TF cofactor function is selectively substituted, deleted or replaced to result in a molecule which retains its ability to bind FVII/FVIIa but which has reduced ability to act as a cofactor as described above.

## 2. Active Site Domain

According to the present invention the "active site inhibitor domain" of the compositions may be selected from any one of several groups of Factor VIIa active site directed inhibitors. Such active site inhibitors are broadly categorized for the purpose of the present invention into i) active site inhibitors which reversibly bind the active site of FVIIa and are cleavable by Factor VIIa, ii) active site inhibitors which reversibly bind to the active site of Factor VIIa but cannot be cleaved, and iii) active site inhibitor molecules which irreversibly bind to the active site of Factor VIIa. For a review of inhibitors of serine proteinases see, Proteinase Inhibitors (Research Monographs in Cell and Tissue Physiology; v. 12) Elsevier Science Publishing Co., Inc., New York (1990).

According to the first group, active site inhibitor domains which reversibly bind to and are cleavable by the active site of Factor VIIa, the Kunitz and Kazal type serine protease inhibitors are preferred with the Kunitz type being most preferred. Reversible active site inhibitor domains bind to the active site of Factor VIIa through non-covalent interactions such as ionic bonds, hydrophobic interactions or hydrogen bonding. Kunitz domains are recognized by the skilled artisan to be slow tight binding reversible inhibitors of serine proteases.

Kunitz domain active site inhibitor domains of the present invention include any of the known mammalian Kunitz type domains and especially the Kunitz domain APPI (from human Alzheimer's disease amyloid  $\beta$ -protein precursor; Castro, M. et al. (1990) FEBS Lett. 267:207-212) residues 1-58; TFPI-KD1 (residues 22-79), TFPI-KD2 (residues 93-150), and TFPI-KD3 (residues 185-242) of human TFPI (tissue factor protein inhibitor or LACI, Broze Jr., G. J. et al., (1990) Biochemistry 29:7539-7546); ITI-KD1 and ITI-KD2, (residues 22-79 and 78-135) of human inter- $\alpha$ -trypsin inhibitor, respectively (Vetr, H. et al., (1989) FEBS Lett. 245:137-140); Collagen  $\alpha$  3 (VI) (residues 2899-2956) Collagen alpha 3 (VI) chain precursor (Chu, M. L. (1990) et al. EMBO J. 9:385-393); HKIB9 (7-60) Human Kunitz-type protease inhibitor, HKIB9 (Norris, K., in Genbank Database (Dec 31, 1993, Release 39.0), submitted Jan 19, 1994); BPTI (1-58), Aprotinin, bovine basic pancreatic trypsin inhibitor (Creighton T. E. and Charles, I. G., (1987) Cold Spring Harbor Symp. Quant. Biol. 52:511-519).

Preferred among the Kunitz-type serine proteases are those molecules which bind FVIIa active site with sufficient affinity to reversibly inhibit the molecule. By this is meant that the Kunitz-type domain is able to bind to and inhibit the catalytic activity of FVIIa as measured by routine assays such as those described (below). Such molecules have an apparent dissociation constant ( $K_d$ ) with respect to TF-FVIIa of less than about 100 nM,

Preferred among the TF domains of the present invention are those TF domains wherein all or a portion of the transmembrane and cytoplasmic domains of wild type tissue factor have been deleted. According to this aspect of the present invention, the TF domain comprises at least a portion of the N-terminal fibronectin type III domain of wild type tissue factor. Preferably, the TF domain comprises at least amino acids 1-102 of wild type tissue factor. More preferably the TF domain of the present invention comprises both fibronectin type III domains of wild type tissue factor. Preferably, according to this aspect of the present invention, at least amino acids 1-219 of wild type TF are present.

The TF domain of the present invention comprises a polypeptide which retains its ability to bind FVII/FVIIa and which is further capable of neutralizing the procoagulant activity of wild type tissue factor as described above. By way of illustration, substitution, insertion or deletions of particular amino acids along the length of wild type TF produce TF variants with reduced ability to act as a cofactor for FVIIa. The skilled artisan will recognize those residues of wild type TF which contribute to the procoagulant function of TF. For example, residues in the area of amino acids 157-168 contribute to the procoagulant function of TF-FVIIa (Kelley et al., (1995) *supra*; Ruf et al., (1992) *supra*) but are not important for FVII/FVIIa binding. According to the present invention any or all of these amino acids are selectively substituted or deleted to provide a TF domain that binds to FVII/FVIIa but is capable of neutralizing the procoagulant activity of wild type tissue factor.

In a preferred embodiment, any or all of residues Trp158, Lys159, Ser163, Gly164, Lys165, Lys166, and Tyr185 of wild type tissue factor are selectively substituted or deleted to provide a TF domain of the present invention. Preferred substitutions are described in U.S. Patent No. 5,346,991 and include substitution with an amino acid other than one bearing a substantially positively charged side chain at physiological pH. Exemplary substitutions include any or all of Trp158Phe, Lys159Ala, Ser163Ala, Lys165Ala, Lys166Ala, and Tyr185Ala. In a most preferred aspect of the present invention, lysine residues 165 and 166 which are important to TF cofactor function but do not interfere with FVIIa complex formation (Roy et al., (1991) *J. Biol. Chem.* 266:22063; Ruf et al., (1992) *J. Biol. Chem.* 267:6375) are selectively substituted. Therefore, according to a preferred aspect of the present invention at least residues 165 and 166 of wild type tissue factor are selectively substituted to result in a molecule which retains its ability to bind FVII/FVIIa but has a reduced ability to act as a cofactor as described.

In a particular aspect, alanine substitution of these residues is preferred although any substitution which results in a decreased rate of FX activation catalyzed by the TF-FVIIa complex (Ruf et al., (1992) *supra*) is appropriate. Lysine residues 165 and 166 are located on the C-terminal fibronectin type III domain of TF on the opposite surface of the molecule from residues found to be important for FVIIa binding on the basis of mutagenesis results (Kelley et al., (1995) *supra*).

Preferred tissue factor domains of the present invention are those described in U.S. Patent No. 5,346,991, entitled "Tissue Factor Mutants Useful for the Treatment of Myocardial Infarction and Coagulopathic Disorders" the disclosure of which is specifically incorporated herein by reference. This patent describes the generation of tissue factor variants that are capable of inhibiting the ability of endogenous tissue factor to induce coagulation. These variants have either or both of the positively charged amino acid residues 165 and 166 substituted with an  $\alpha$ -amino acid other than one bearing a substantially positively charged side chain at physiological pH. The variants include human tissue factor molecules as described above having the cytoplasmic

R<sub>3</sub> is selected from the group YGG and YSG.

R<sub>4</sub> is selected from the group APPI amino acids 40-58 (Met52Ala), GNRNNFDTEEYCAA VCGSA (SEQ ID NO: 18); APPI amino acids 40-58, GNRNNFDTEEYCM A VCGSA (SEQ ID NO: 19); TFPI-KD1 amino acids 61-79, GNQNR FESLEECKKMCTRD (SEQ ID NO: 20); TFPI-KD2 amino acids 132-150,  
 5 GNMNNFETLEECKNICEDG (SEQ ID NO: 21); TFPI-KD3 amino acids 224-242, GNENNFTSKQECLRACKKG (SEQ ID NO: 22); ITI-KD1 amino acids 61-79, GNGNNFVTEKECLQTCRTV (SEQ ID NO: 23); ITI-KD2 amino acids 117-135, GNGNKFYSEKECREYCGVP (SEQ ID NO: 24); Collagen α 3(VI) amino acids 2938-2956, GNENKFGSQKECEKVCAPV (SEQ ID NO: 25); HK1B9 amino acids 40-58, GNSNNFLRKEKCEKCKFT (SEQ ID NO: 26); and BPTI amino acids 40-58, AKRNNFKSAEDCMRTCGGA  
 10 (SEQ ID NO: 27);

In a preferred Kunitz type active site inhibitor domain R<sub>1</sub> has the sequence VREVCSEQAE (SEQ ID NO: 1) R<sub>2</sub> has the sequence RWYFDVTEGKCAPF (SEQ ID NO: 9) R<sub>3</sub> has the sequence YGG; and R<sub>4</sub> has the sequence: GNRNNFDTEEYCAA VCGSA (SEQ ID NO: 18) or GNRNNFDTEEYCM A VCGSA (SEQ ID NO: 19).

15 Therefore, preferred Kunitz type active site inhibitor domains include:

I-18	R <sub>1</sub> PGVCRALILR <sub>2</sub> FR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 28)
I-49	R <sub>1</sub> PGWCRALILR <sub>2</sub> FR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 29)
I-14	R <sub>1</sub> PGFCRALILR <sub>2</sub> FR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 30)
I-16	R <sub>1</sub> GGWCRALILR <sub>2</sub> FR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 31)
20	where R <sub>4</sub> is the sequence identified by SEQ ID NO: 19, and	
II-4	R <sub>1</sub> PGPCRAMISR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 32)
II-3	R <sub>1</sub> PGWCRAMISR <sub>2</sub> IR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 33)
II-6	R <sub>1</sub> PGPCKAMISR <sub>2</sub> IR <sub>3</sub> CWR <sub>4</sub>	(SEQ ID NO: 34)
III-27	R <sub>1</sub> TGPCRALISR <sub>2</sub> WR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 35)
25	III-30	R <sub>1</sub> TGPCRALISR <sub>2</sub> YR <sub>3</sub> CGR <sub>4</sub> (SEQ ID NO: 36)
TF7I-VY	R <sub>1</sub> PGVCRALILR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 37)
TF7I-LY	R <sub>1</sub> PGLCRALILR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 38)
TF7I-WY	R <sub>1</sub> PGWCRALILR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 39)
TF7I-PG	R <sub>1</sub> PGPCRALILR <sub>2</sub> FR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 40)
30	IV-47C	R <sub>1</sub> PGPCRAMMKR <sub>2</sub> IR <sub>3</sub> CHR <sub>4</sub> (SEQ ID NO: 41)
IV-54C	R <sub>1</sub> PGPCRALMKR <sub>2</sub> VR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 42)
IV-31B	R <sub>1</sub> PGPCRALMKR <sub>2</sub> VR <sub>3</sub> CFR <sub>4</sub>	(SEQ ID NO: 43)
IV-49C	R <sub>1</sub> PGPCRAMMKR <sub>2</sub> IR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 44)
IV-50C	R <sub>1</sub> PGPCRAMYKR <sub>2</sub> IR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 45)
35	IV-57C	R <sub>1</sub> PGVCRAMMKR <sub>2</sub> IR <sub>3</sub> CGR <sub>4</sub> (SEQ ID NO: 46)
IV-51C	R <sub>1</sub> PGPCKALMRR <sub>2</sub> YR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 47)
IV-35B	R <sub>1</sub> PGPCKAIMKR <sub>2</sub> IR <sub>3</sub> CHR <sub>4</sub>	(SEQ ID NO: 48)
IV-58C	R <sub>1</sub> PGPCKALMKR <sub>2</sub> YR <sub>3</sub> CHR <sub>4</sub>	(SEQ ID NO: 49)
IV-48C	R <sub>1</sub> PGPCKALMKR <sub>2</sub> WR <sub>3</sub> CWR <sub>4</sub>	(SEQ ID NO: 50)

more preferably the molecules have an apparent dissociation constant of less than about 10 nM. Most preferred among the Kunitz type domains are those that have a  $K_i^*$  of less than about 5 nM for TF-Factor VIIa.

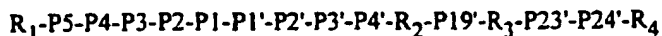
- Apparent equilibrium dissociation constants ( $K_i^*$ ) are determined using methods derived for tight-binding inhibitors (Bieth, J., (1974) *Proteinase Inhibitors* 463-469; Williams, J. W. and Morrison, J. F., (1979) *Methods Enzymol* 63:437-467), assuming enzyme and inhibitor form a reversible complex with a 1:1 stoichiometry as has been observed for the interaction of Kunitz-type domain with the FVIIa serine protease (Bode, W. and R. Huber, (1992) *Eur. J. Biochem.* 204:433-451; Laskowski, M., Jr. and I. Kato, (1980) *Annu. Rev. Biochem.* 49:593-626). The data are fit by nonlinear regression analysis to Equation 1:

$$V_i/V_o = 1 - \frac{[E_o] + [I_o] + K_i^* - \sqrt{([E_o] + [I_o] + K_i^*)^2 - (4 \cdot [E_o] \cdot [I_o])}}{2 \cdot [E_o]} \quad (1)$$

- where  $V_i/V_o$  is the fractional activity (steady-state inhibited rate divided by the uninhibited rate),  $[E_o]$  is the total FVIIa concentration and  $[I_o]$  is the total inhibitor concentration.

- By way of example and not limitation, preferred Kunitz type domains have a 58 amino acid backbone structure, with amino acids 11-19 corresponding to primary binding loop which fits into the active site of the serine protease. Kunitz-type domains having a particular amino acid sequence within the primary binding loop demonstrate preferred inhibition as defined above. Exemplary Kunitz domains showing the preferred inhibition of factor VIIa as described above are Kunitz type domains with a Pro at position P5, a Gly at position P4, a Pro, Val, Leu, or Trp at position P3, a Cys at position P2, an Arg, Lys, or Met at position P1, an Ala at position P1', a Leu, Met or Ile at position P2', a Met, Ile, Leu or Tyr at position P3', and Leu, Lys or Arg at position P4'. Kunitz type domains also preferred according to the present invention also feature Phe, Val, Ile, Trp, or Tyr at position 34 (P19') a Cys at position 38 (P23') and a His, Tyr, or Gly at position 39 (P24').

Exemplary Kunitz type active site inhibitor domains are also described in International Publication No. WO 95/23860 and are represented by structural formula I:



- According to this formula, when P5-P4', P19', P23', and P24' are selected as described above,  $R_1$  is selected from APPI amino acids 1-10, VREVCSEAE (SEQ ID NO: 1); TFPI-KD1 amino acids 22-31, MHSFCAFKAD (SEQ ID NO: 2); TFPI-KD2 amino acids 93-102, KPDFCFLEED (SEQ ID NO: 3); TFPI-KD3 amino acids 185-194, GPSWCLTPAD (SEQ ID NO: 4); ITI-KD1 amino acids 22-31, KEDSCQLGYS (SEQ ID NO: 5); ITI-KD2 amino acids 78-87, TVAACNLPIV (SEQ ID NO: 6); HKIB9 amino acids 1-10, LPNVCAFPME (SEQ ID NO: 7); and BPT1 amino acids 1-10, RPDFCLEPPY (SEQ ID NO: 8).

- $R_2$  is selected from APPI amino acids 20-33, RWYFDVTEGKCAPF (SEQ ID NO: 9); TFPI-KD1 amino acids 41-54, RFFNFIFTRQCEEF (SEQ ID NO: 10); TFPI-KD2 amino acids 112-125, RYFYNNQTKQCERF (SEQ ID NO: 11); TFPI-KD3 amino acids 204-217, RFYYNSVIGKCRPF (SEQ ID NO: 12); ITI-KD1 amino acids 41-54, RYFYNGTSMACETF (SEQ ID NO: 13); ITI-KD2 amino acids 97-110, LWAFDAVKGKCVLF (SEQ ID NO: 14); Collagen  $\alpha$  3(VI) amino acids 2918-2931, KWYYDPNTKSCARF (SEQ ID NO: 15); HKIB9 amino acids 20-33, RWFFNFETGECELF (SEQ ID NO: 16); and BPT1 amino acids 20-33, RYFYNAKAGLCQTF (SEQ ID NO: 17).

variable length. The amino acid composition of the polypeptide determines the character and length of the linker. In a preferred embodiment, the linker molecule comprises a flexible, hydrophilic polypeptide chain. Exemplary linker domains comprises one or more [(Gly)<sub>4</sub>-Ser] units.

In a preferred aspect, the active site inhibitor domain is a Kunitz type serine protease inhibitor, the TF domain is hTFAA and the linker domain is a polypeptide comprising between 5 and about 35 amino acids and spanning a distance of greater than about 30Å. More preferably, the linker comprises between about 20 and about 30 amino acids and spans a distance of greater than about 60Å. Most preferably the linker comprises about a 22 to 27 amino acid polypeptide and is comprised of about 4 to 5 [(Gly)<sub>4</sub>-Ser] units.

#### 4. Synthesis

The hybrid molecules of the present invention may be synthesized by various techniques which are well known in the art. These include recombinant DNA techniques, solid phase synthesis, solution phase synthesis, organic chemical synthetic techniques or a combination of these techniques. The choice of synthesis will depend upon the particular molecule to be generated. For example, in one embodiment the hybrid molecule of the present invention is not entirely "protein" in nature and is synthesized by a combination of recombinant techniques and solution phase techniques.

For example, for the synthesis of hybrid molecules of the present invention which contain both amino acid or protein portions and non-protein portions the molecule is generally obtained by synthesizing the protein portion of the molecule by recombinant techniques as described below followed by coupling the non protein portion of the molecule to the protein portion via solid phase or solution phase methods.

For the coupling of protein and non protein portions of the molecule, chemical coupling is preferred. According to this procedure, traditional crosslinking agents are employed such as molecules having one or more reactive groups that can react with more than one sites on the protein and non-protein molecules. Biocompatible crosslinkers are preferred having the characteristics of being non-carcinogenic, nontoxic, and substantially nonimmunogenic *in vivo*. Typically the cross linker will covalently bond with an amino group or a sulfhydryl group on the protein portion of the molecule and a hydroxyl, amino, aldehyde or carboxylic acid group of the non-protein portion of the molecule. Methods of crosslinking molecules are reviewed by Means and Feaney (1990) Bioconjugate Chemistry, 1:2-12.

Various techniques are available which may be employed to produce DNA, which can encode proteins for the recombinant synthesis of hybrid proteins of the invention. For instance, it is possible to derive DNA based on naturally occurring DNA sequences that encode for changes in an amino acid sequence of the resultant protein. These mutant DNA can be linked together and used to obtain the hybrid molecules of the present invention. These techniques contemplate, in simplified form, obtaining a gene encoding a tissue factor domain and a gene encoding an active site inhibitor domain such as a Kunitz type domain of structural formula I; modifying the genes by recombinant techniques such as those discussed below; inserting the genes into an appropriate expression vector separated by a gene encoding a linker module of various lengths; inserting the vector into an appropriate host cell; culturing the host cell to cause expression of the hybrid molecule; and purifying the molecule produced thereby.



	IV-46C	R <sub>1</sub> PGPCKAMIKR <sub>2</sub> LR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 51)
	IV-55C	R <sub>1</sub> PGPCKALMKR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 52)
	IV-32B	R <sub>1</sub> PGPCKALMKR <sub>2</sub> YR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 53)
	IV-36B	R <sub>1</sub> PGPCKALMKR <sub>2</sub> VR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 54)
5	IV-40B	R <sub>1</sub> PGACKAMYKR <sub>2</sub> IR <sub>3</sub> CGR <sub>4</sub>	(SEQ ID NO: 55)
	53b	R <sub>1</sub> PGPGRALILR <sub>2</sub> FR <sub>3</sub> AYR <sub>4</sub>	(SEQ ID NO: 56), and
	TF7I-C	R <sub>1</sub> PGPCRALILR <sub>2</sub> FR <sub>3</sub> CYR <sub>4</sub>	(SEQ ID NO: 57)

where R<sub>4</sub> has the sequence identified by SEQ ID NO: 18.

It will be appreciated by the skilled artisan that with a Kunitz type active site inhibitor domain all or a portion of the Kunitz type domain may be used as the active site inhibitor domain according to the present invention. Any portion of the Kunitz domain which binds with sufficient affinity to reversibly inhibit FVIIa will be sufficient as Factor VIIa active site domain. Such molecules generally comprise at least the P<sub>1</sub> and P<sub>1</sub>' residues of the Kunitz domain and preferably the primary binding loop of the Kunitz domain P<sub>5</sub> through P<sub>4</sub>'.

The active site inhibitor moiety of the present invention may also be an irreversible FVIIa serine protease inhibitor. Such irreversible active site inhibitor domains generally form covalent bonds with the Factor VIIa active site. Such irreversible inhibitors include general serine protease inhibitors such as peptide chloromethylketones (see, Williams et al., (1989) J. Biol. Chem. 264:7536-7540) or peptidyl chloromethanes; azapeptides; acylating agents such as various guanidinobenzoate derivatives and the 3-alkoxy-4-chloroisocoumarins; sulphonyl fluorides such as phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), tosylpropylchloromethyl ketone (TPCK) and tosylsilylchloromethylketone (TLCK); nitrophenylsulphonates and related compounds; heterocyclic protease inhibitors such as isocoumarins, and coumarins.

### 3. Linker

According to the present invention, the tissue factor domain is linked, preferably via its N-terminus to the FVII/FVIIa active site inhibitor domain of the hybrid molecule via a flexible linker domain. The linker component of the hybrid molecule of the invention does not necessarily participate in the binding of the molecule to FVII/FVIIa. Therefore, according to the present invention, the linker domain, is any group of molecules that provides a spatial bridge between the FVII/FVIIa active site inhibitor domain and the tissue factor domain of the molecule.

The linker domain can be of variable length and makeup, however, according to the present invention, it is the length of the linker domain and not its structure that is important. The linker domain preferably allows for the active site domain of the hybrid molecule to fit into the serine protease active site groove of FVII/FVIIa while allowing the tissue factor domain of the molecule to bind to the TF binding site on the FVII/FVIIa molecule. Therefore, the length of the linker domain is dependent upon the character of the two "functional" domains of the hybrid molecule.

One skilled in the art will recognize that various combinations of atoms provide for variable length molecules based upon known distances between various bonds (Morrison, and Boyd, Organic Chemistry, 3rd Ed, Allyn and Bacon, Inc., Boston, MA (1977)). For example, the linker domain may be a polypeptide of

as *E. coli* B, *E. coli* X1776 (ATCC No. 31537), and *E. coli* c600 and c600hfl, *E. coli* W3110 (F-, gamma-prototrophic /ATCC No. 27325), bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella -typhimurium* or *Serratia marcesans*, and various pseudomonas species. The preferred prokaryote is *E. coli* W3110 (ATCC 27325). When expressed by prokaryotes the polypeptides typically contain an N-terminal methionine or a formyl methionine and are not glycosylated. These examples are, of course, intended to be illustrative rather than limiting.

In addition to prokaryotes, eukaryotic organisms, such as yeast cultures, or cells derived from multicellular organisms may be used. In principle, any such cell culture is workable. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a reproducible procedure (Tissue Culture, Academic Press, Kruse and Patterson, eds. [1973]). Examples of such useful host cell lines are VERO and HeLa cells, Chinese Hamster Ovary (CHO) cell lines, WI38, 293, BHK, COS-7 and MDCK cell lines. Yeast expression systems have been used to make Kunitz domains (Wagner, S. L. et al., (1992) Biochem. Biophys. Res. Commun. 186:1138-1145; Vedvick, T. et al., (1991) J. Indust. Microbiol. 7:197-202). In particular the yeast *Pichia pastoris* has been used successfully using the *Saccharomyces cerevisiae*  $\alpha$  mating factor prepro signal sequence and the *P. pastoris* alcohol oxidase AOX1 promoter and terminator sequences. Other yeast expression vectors and hosts commonly used to express heterologous proteins are also contemplated.

### 5. Compositions

The hybrid molecule of the present invention comprising a TF domain and a FVIIa active site domain is typically provided in a compositional form that is suitable for its intended use. The hybrid molecule of the present invention comprising a TF domain can be prepared in the soluble form such as the hTFAA form described herein. According to this aspect of the invention the tissue factor domain of the molecule is prepared without a membrane anchor. Alternatively, the tissue factor domain can be prepared as a full-length membrane-associated form, with the membrane anchor present.

The hybrid molecule of the present invention may also comprise a TF domain comprising all or a portion of the transmembrane domain of wild type tissue factor. It is preferred, according to the present invention, that bifunctional molecules comprising a TF domain containing a membrane anchor domain be formulated in a composition comprising a mild detergent or phospholipid (PL). Although the composition of the present invention comprising a full-length TF domain including a membrane anchor or transmembrane domain retain their biological activity they are preferably formulated in a phospholipid composition. International Publication No. WO 94/28017 describes the preparation of phospholipid compositions comprising a TF domain that are appropriate for the compositions of the present invention.

Preferred compositions described in WO 94/28017 and suitable for the pharmaceutical compositions of the present invention are phospholipid compositions which afford maximum stability and biological activity for the composition. Such phospholipid compositions are preferably formulated to form liposome compositions, as are generally well known in the art. As described, suitable phospholipids for use in the liposome compositions of the present invention include those which contain fatty acids having twelve to twenty carbon atoms; said fatty acids may be either saturated or unsaturated. Preferred phospholipids for use according to the present invention

Somewhat more particularly, a DNA sequence encoding the bifunctional molecule of the present invention is obtained by synthetic construction of the DNA sequence (Sambrook, J. et al., *Molecular Cloning* (2nd ed.), Cold Spring Harbor Laboratory, N.Y., (1989).

By way of example, expression vectors encoding wild type tissue factor can be obtained and subject to site specific mutagenesis (Kunkel et al., (1991) *Methods Enzymol.* 204:125-139; Carter, P., et al., (1986) *Nucl. Acids. Res.* 13:4331; Zoller, M. J. et al., (1982) *Nucl. Acids Res.* 10:6487), cassette mutagenesis (Wells, J. A., et al., (1985) *Gene* 34:315), or restriction selection mutagenesis (Wells, J. A., et al., (1986) *Philos. Trans, R. Soc. London Ser A* 317, 415) to obtain the tissue factor domain of the molecule. The mutant DNA can then be used by insertion into expression vectors containing DNA encoding an active site inhibitor domain. A DNA sequence encoding a linker domain of varying length such as one or more [(Gly)<sub>4</sub>-Ser] units can then be inserted into the same expression vector.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing the DNA encoding the tissue factor variants and the Kunitz type active site inhibitor domains of the present invention. This technique is well known in the art as described by Adelman et al., (1983) *DNA*, 2:183. Briefly, the native or unaltered DNA of a wild type tissue factor or Kunitz type domain, for instance APPI, is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence.

The DNA encoding variants are then inserted into an appropriate plasmid or vector separated by DNA sequence encoding a linker domain. The vector is used to transform a host cell. In general, plasmid vectors containing replication and control sequences which are derived from species compatible with the host cell are used in connection with those hosts. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells.

For example, *E. coli* may be transformed using pBR322, a plasmid derived from an *E. coli* species (Mandel, M. et al., (1970) *J. Mol. Biol.* 53:154). Plasmid pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides easy means for selection. Other vectors include different features such as different promoters, which are often important in expression. For example, plasmids pKK223-3, pDR720, and pPL-lambda represent expression vectors with the tac, trp, or P<sub>L</sub> promoters that are currently available (Pharmacia Biotechnology).

Other preferred vectors can be constructed using standard techniques by combining the relevant traits of the vectors described herein. Relevant traits of the vector include the promoter, the ribosome binding site, the variant gene or gene fusion, the signal sequence, the antibiotic resistance markers, the copy number, and the appropriate origins of replication.

In *E. coli*, Kunitz domains have been expressed as intact secreted proteins (Castro, M. et al., (1990) *FEBS Lett.* 267:207-212), intracellularly expressed proteins (Altman, J. D. et al., (1991) *Protein Eng.* 4:593-600), or as fusion proteins (Sinha, S. et al., (1991) *J. Biol. Chem.* 266:21011-21013; Lauritzen, C. et al., (1991) *Prot. Express. Purif.* 2:372-378; Auerswald, E. A. et al., (1988) *Biol. Chem. Hoppe-Seyler* 369:27-35).

The host cell may be prokaryotic or eukaryotic. Prokaryotes are preferred for cloning and expressing DNA sequences to produce parent polypeptides, segment substituted polypeptides, residue-substituted polypeptides and polypeptide variants. For example, *E. coli* K12 strain 294 (ATCC No. 31446) may be used

## 6. Therapeutic methods

The bifunctional molecules of the present invention can be used therapeutically to prevent the biological activity of the TF-FVIIa complex. The inhibition of TF-FVIIa is desirable in indications where the reduction of TF-FVIIa dependent coagulation is implicated. These situations include but are not limited to the prevention of arterial rethrombosis in combination with thrombolytic therapy. It has been suggested that the TF-FVIIa plays a significant role in a variety of clinical states including deep venous thrombosis, arterial thrombosis, stroke, DIC, septic shock, cardiopulmonary bypass surgery, adult respiratory distress syndrome, hereditary angioedema. Inhibitors of TF-FVIIa may therefore play important roles in the regulation of inflammatory and/or thrombotic disorders.

Thus the present invention encompasses a method for preventing TF-FVIIa mediated event in a human comprising administering to a patient in need thereof a therapeutically effective amount of the hybrid molecule of the present invention. A therapeutically effective amount of the hybrid molecule of the present invention is predetermined to achieve the desired effect. The amount to be employed therapeutically will vary depending upon therapeutic objectives, the routes of administration and the condition being treated. Accordingly, the dosages to be administered are sufficient to bind to available FVII/FVIIa and form an inactive complex leading to decreased coagulation in the subject being treated.

The therapeutic effectiveness is measured by an improvement in one or more symptoms associated with the TF-FVIIa dependant coagulation. Such therapeutically effective dosages can be determined by the skilled artisan and will vary depending upon the age condition, sex and condition of the subject being treated. Suitable dosage ranges for systemic administration are typically between about 1 µg/kg to up to 100 mg/kg or more and depend upon the route of administration. According to the present invention a preferred therapeutic dosage is between about 1 µg/kg body weight and about 5 mg/kg body weight. For example, suitable regimens include intravenous injection or infusion sufficient to maintain concentration in the blood in the ranges specified for the therapy contemplated.

Pharmaceutical compositions which comprise the polypeptides of the invention may be administered in any suitable manner, including parental, topical, oral, or local (such as aerosol or transdermal) or any combination thereof. Suitable regimens also include an initial administration by intravenous bolus injection followed by repeated doses at one or more intervals.

Where the composition of the invention is being administered in combination with a thrombolytic agent, for example, for the prevention of reformation of an occluding thrombus in the course of thrombolytic therapy, a therapeutically effective dosage of the thrombolytic is between about 80 and 100 % of the conventional dosage range. The conventional dosage range of a thrombolytic agent is the daily dosage used in therapy and is readily available to the treating physician. (Physicians Desk Reference 1994, 50th Edition, Edward R. Barnhart, publisher). The typical dosage range will depend upon the thrombolytic being employed and include for tissue plasminogen activator (t-PA), 0.5 to about 5 mg/kg body weight; streptokinase, 140,000 to 2,500,000 units per patient; urokinase, 500,000 to 6,250,00 units per patient; and anislated streptokinase plasminogen activator complex (ASPAC), 0.1 to about 10 units/ kg body weight.

The term combination as used herein includes a single dosage form containing at least the molecule of the present invention and at least one thrombolytic agent. The term is also meant to include multiple dosage

include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS). These phospholipids may come from any natural source and the phospholipids, as such, may be comprised of molecules with differing fatty acids. Phospholipid mixtures comprising phospholipids from different sources may be used. For example, PC, PG and PE may be obtained from egg yolk; PS may be  
5 obtained from animal brain and spinal chord. These phospholipids may come from synthetic sources as well. The phospholipids are conveniently combined in the appropriate ratios to provide the phospholipid mixture for use in preparing the composition of the present invention.

The preparation of liposomes is generally well known and has been previously described. Exemplary methods for preparation of liposomes includes reverse loading of liposomes (see U.S. Pat. No. 5,104,661), or  
10 in the manner described for the incorporation of amphotericin B into lipid vesicles. (See, e.g., Lopez-Berenstein et al., (1985) J. Infect. Dis., 151:704-710; Lopez-Berenstein, (1987) Antimicrob. Agents Chemother., 31:675-678; Lopez-Berenstein et al., (1984) J. Infect. Dis., 150:278-283; and Mehta et al., (1984) Biochem. Biophys. Acta, 770:230-234). Liposomes with enhanced circulation time may also be prepared as described in U.S. Pat. No. 5,013,556.

15 Thus, in one embodiment, the present invention contemplates the preparation of the hybrid molecules in the form of liposomes having TF domain portion of the molecule associated with the lipid bilayer of the liposomes, such that the TF membrane anchor domain is inserted through the lipid bilayer.

Other suitable compositions of the present invention comprise any of the above noted compositions with a pharmaceutically acceptable carrier, the nature of the carrier differing with the mode of administration, for  
20 example, in oral administration, usually using a solid carrier and in I.V. administration, a liquid salt solution carrier.

The compositions of the present invention include pharmaceutically acceptable components that are compatible with the subject and the protein of the invention. These generally include suspensions, solutions and elixirs, and most especially biological buffers, such as phosphate buffered saline, saline, Dulbecco's Media, and  
25 the like. Aerosols may also be used, or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like (in the case of oral solid preparations, such as powders, capsules, and tablets).

As used herein, the term "pharmaceutically acceptable" generally means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized  
30 pharmacopeia for use in animals, and more particularly in humans.

The formulation of choice can be accomplished using a variety of the aforementioned buffers, or even excipients including, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, and the like. "PEGylation" of the compositions may be achieved using techniques known to the art (see for example International Patent Publication No. WO92/16555,  
35 U.S. Patent No. 5,122,614 to Enzon, and International Patent Publication No. WO92/00748). Oral compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders.

with NsiI which cuts the DNA at the junction between the stII and hTFAA segments. The NsiI cut plasmid was ligated with the PstI/BamHI Kunitz domain fragment and two oligonucleotide linkers prepared by chemical synthesis. One linker had Nsi/PstI cohesive ends thus joining the Kunitz domain in frame with the stII signal sequence. The second linker has BamHI/NsiI cohesive ends, encodes a single G<sub>4</sub>S module, and joins the Kunitz domain in frame with the N-terminus of hTFAA. The stop codon is derived from pZTFAA. Fusion genes having multiple copies of the G<sub>4</sub>S module were constructed by inserting chemically synthesized oligonucleotides encoding the (G<sub>4</sub>S)<sub>n</sub> unit between the appropriate restriction enzyme sites in TFKD1 or KDTF1. For TFKD1, the SacII/PstI sites were used, and for KDTF1, the BamHI/NsiI sites were used. All fusion genes were confirmed by dideoxynucleotide sequencing (Sanger, F. et al., PNAS(USA) (1977)74:5463-5467).

Phagemids encoding the fusion proteins were transformed into *E. coli* strain 33B6, a derivative of *E. coli* W3110, for expression. Overnight saturated cultures were used to inoculate (1%) 10 L of media in a fermentation tank. Fermentation was performed as described previously (Carter, P. et al., Bio/Technology, (1992)10:163-167) except that the temperature was 30 °C rather than 37 °C. Fusion proteins were secreted into the periplasm by virtue of the stII signal sequence. Cells were harvested by centrifugation 32 hours after inoculation and stored frozen at -20 °C.

## Example 2

### Purification of Fusion Proteins

Fusion proteins were extracted from *E. coli* cell paste and purified by immunoaffinity chromatography on an anti-TF monoclonal antibody (D3) column (Paborsky, L. R. et al., Biochemistry (1989) 28:8072-8077) as described for mutants of soluble tissue factor (Kelley, R. F. et al., (1995) Biochemistry 34:10383-10392). In some cases, a final size exclusion chromatography step, using a Superdex 75 (Pharmacia) column equilibrated with phosphate-buffered saline, was incorporated to purify fusion protein monomers from higher mass oligomers. The course of the fusion protein purification was followed by SDS-PAGE as shown in Figure 4. This procedure yielded highly purified fusion protein. Each purified sample was submitted for analysis on a Sciex API 3 mass spectrometer equipped with an articulated electrospray source to verify that protein mass was consistent with the desired molecular composition. Multiply charged ions of horse myoglobin (MW = 16,951 Da) were used for instrument calibration. Results of the analysis by mass spectrometry are shown in Table I and indicate that the fusion proteins have the expected molecular composition. Concentrations of the purified fusion proteins were determined by three methods (Table I): 1) Detection of hTFAA with the D3 antibody, 2) determination of Kunitz domain by titration of trypsin, and 3) absorbance measurements to determine total protein concentration. All three methods gave similar concentrations. Taken together with the mass spectrometry data these results show that the fusion proteins are intact and that both the Kunitz domain and TF domains are functional when present in the fusion protein.

The fusion proteins tested are listed below in Table I using shorthand notation KDTFn or TFKDn, wherein n is an integer referring to the number of G<sub>4</sub>S linker units. According to that notation TF refers to hTFAA. KD refers to the TF71-c Kunitz domain. KD following TF (TFKD1) refers to a fusion protein wherein the KD domain is linked at its N-terminus via the peptide linker to the C-terminus of hTFAA. TF following KD

forms wherein the molecule of the present invention is administered separately but concurrently by two separate administration, such as in sequential administration. These combinations and compositions work to dissolve or prevent the formation of an occluding thrombus resulting in dissolution of the occluding thrombus.

According to a further aspect of the invention the hybrid molecule may be employed in preventing ex vivo coagulation such as that encountered in the extracorporeal perfusion of blood through for example artificial valves, prothesis, stents or catheters. According to this aspect of the invention the extracorporeal devise may be coated with the compositions of the invention resulting a lower risk of clot formation due to extrinsic pathway activation.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are expressly incorporated herein by reference.

## EXAMPLES

### Materials

Human Factor VIIa, Factor X, Factor Xa, were purchased from Haematologic Technologies Inc. (Essex Jct., VT). Membrane tissue factor (mTF) was prepared by sonication of a human embryonic kidney cell line (293) expressing recombinant, full length (residues 1-263) human TF (Paborsky, L. R. et al., (1990) Protein Engineering 3:547-553). Bovine trypsin, 4-methylumbelliferyl p-guanidinobenzoate and CHAPS were purchased from Sigma Chemicals, Inc. Bovine serum albumin (BSA), Fraction V was obtained from Calbiochem (La Jolla, CA). N<sup>α</sup>-Benzoyl-L-arginine-*p*-nitroanilide was purchased from Bachem California (Torrance, CA). Human plasmin, S-2302, S-2251 and S-2366 were purchased from Kabi Vitrum (Sweden) and SPECTROZYME™ FXa was purchased from American Diagnostica (Greenwich, CT) and CHROMOZYME™ t-PA was from Boehringer Mannheim. All other reagents were of the highest grade commercially available.

### Example 1

#### Construction and Expression of Fusion Proteins

Plasmids encoding tissue factor-Kunitz domain fusion proteins were constructed from the plasmid pZTFAA (Kelley, R. F. et al., (1995) Biochemistry 34:10383-10392) designed for expression of hTFAA by secretion from *E. coli*. As shown in Figure 3, the expression unit of pZTFAA has the stII signal sequence joined to the N-terminus of hTFAA and transcription is driven by the alkaline phosphatase promoter (phoA). pZTFAA also has elements necessary for replication of the plasmid in single or double stranded form. The coding sequence for the TF7I-C Kunitz domain was excised from the plasmid pA4G32-TF7I-C (Dennis, M. S. & Lazarus, R. A. J. Biol. Chem. (1994) 269:22129-22136) by restriction enzyme digest with PstI and BamHI. For construction of genes encoding fusion proteins with the Kunitz domain joined to the C-terminus of hTFAA, a SacII restriction enzyme site was introduced into pZTFAA by oligonucleotide-directed mutagenesis (Kunkel, T.A. et al., Methods in Enzymology, (1987) 154:367-382). The resulting plasmid was digested with SacII and SphI, and then ligated with the PstI/BamHI fragment of the Kunitz domain and two oligonucleotide linkers prepared by chemical synthesis. One linker has SacII/PstI cohesive ends and encodes a single copy of the G<sub>4</sub>S module and was designed to fuse hTFAA in the correct reading frame with the N-terminus of the Kunitz domain. The second linker has BamHI/SphI cohesive ends and supplies the stop codon of the construct. For construction of fusion protein genes having a Kunitz domain attached to the N-terminus of hTFAA, pZTFAA was cleaved

Activation assays used mTF, FVIIa and FX at final concentrations of 500 pM, 100 pM and 190 nM, respectively, in a volume of 200  $\mu$ L. Inhibitor, if added, was present at a final concentration of 1 nM. This solution also contained 50 mM Tris-HCl pH 8, 150 mM NaCl, and 5 mM  $\text{CaCl}_2$ . The concentration of a stock solution of FVIIa was determined by active site titration with a quantitated sample of TF71-C and by using  
5 CHROMOZYME™ t-PA as the substrate for FVIIa. The concentration of TF71-C had been accurately determined by titration with trypsin that had been active site-titrated using 4-methylumbelliferyl p-guanidinobenzoate (Jameson, G. W. et al., (1973) Biochem. J. 131:107-117). After a 1 h incubation of 80 nM trypsin plus an aliquot of diluted inhibitor in 50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM  $\text{CaCl}_2$ , and 0.05 % TRITON™ X-100 at room temperature, 20  $\mu$ l of 5 mM N-benzoyl-L-arginine-p-nitroanilide was added to a total  
10 volume of 150  $\mu$ l. The change in absorbance at 405 nm was then monitored. The concentrations determined assumed a 1:1 stoichiometry of inhibitor with trypsin or FVIIa. The concentration of mTF was then determined from the increase in the rate of CHROMOZYME™ t-PA hydrolysis upon addition to a solution of the active site quantitated FVIIa. The concentration of FX and FXa was that supplied by the manufacturer.

In tests of the inhibitory properties of the fusion proteins, FVIIa was incubated with the fusion protein  
15 for 1 hour at 37 °C prior to addition of mTF. After adding mTF, incubation at 37 °C was continued for another hour before adding the substrate FX. Timing of the reaction began with substrate addition and 25  $\mu$ L aliquots were removed at various times and mixed with an equal volume of 50 mM EDTA. The amount of FXa formed was measured by adding Factor Xa buffer (10X = 0.2 M HEPES pH 7.4, 1.5 M NaCl, 0.25 M EDTA, 1 % PEG-8000) to a final concentration of 1X followed by 0.5 mM SPECTROZYME™ FXa. The final volume for each  
20 time point was 200  $\mu$ L and the rates of SPECTROZYME™ FXa hydrolysis were monitored by changes in the absorbance at 405 nm and are reported in mOD/min.

The effect of 1 nM inhibitors on FX activation catalyzed by mTF-FVIIa is shown in Figure 5. At a 1 nM concentration of either TF71-C or hTFAA alone, the rate of FXa generation is equivalent to that observed in the uninhibited control. Fusion proteins KDTF1 and TFKD1 also had little effect on the rate of FX activation. In  
25 contrast, KDTF2 gave a slight inhibition of FX activation. The extent of inhibition increased for fusion proteins with a longer linker and complete inhibition was observed with the KDTF5 fusion protein. KDTF4 appeared to be equipotent with KDTF5 whereas KDTF6 and KDTF7 were slightly weaker inhibitors. These data show that a potent inhibitor can be obtained by joining the Kunitz domain and hTFAA domains with a linker of appropriate length. The best linker in this series has 4 or 5  $\text{G}_4\text{S}$  modules corresponding to a polypeptide chain  
30 length, in extended conformation, of 45-60 Å.

#### Example 4

##### Determination of Equilibrium Dissociation Constants

The apparent equilibrium dissociation constant ( $K_i^*$ ) for inhibition of FX activation was determined for hTFAA, TF71-C and KDTF5. These experiments were conducted as described for Example 3 except that the FVIIa and  
35 mTF concentrations were 200 pM and 1 nM, respectively, and the inhibitor concentration was varied. A standard curve was constructed for SPECTROZYME™ FXa hydrolysis by purified FXa (Hematech) such that the observed rate of hydrolysis for each time point could be converted into a concentration of FXa generated. These data were then analyzed by least squares linear regression to calculate the initial velocity of FXa generation for each concentration of inhibitor. Initial velocities were compared to the uninhibited rate to yield a fractional rate



(KDTF) refers to a fusion protein wherein the KD C-terminus is linked via the peptide linker to the N-terminus of the hTFAA.

Table I

Fusion Protein Species	Mass Predicted	Mass Observed	D3 binding-based TF conc.	Trypsin inhibition-based KD conc.	Total protein conc. (A <sub>280</sub> )
TFKD1	31440	31446 ± 5	28 μM	29 μM	28 μM
KDTF1	31511	31515 ± 5	74 μM	77 μM	76 μM
KDTF2	31826	31829 ± 2	66 μM	64 μM	66 μM
KDTF3	32142	32146 ± 4	36 μM	38 μM	36 μM
KDTF4	32457	32456 ± 3	37 μM	35 μM	31 μM
KDTF5	32772	32774 ± 5	20 μM	17 μM	16 μM
KDTF6	33087	33088 ± 4	28 μM	22 μM	39 μM
KDTF7	33403	ND	33 μM	28 μM	47 μM

- TABLE 1. Characterizations of purified fusion proteins. Predicted masses for fusion proteins in their native disulfide-bonded conformations are shown in Column 2. Results of mass spectral analyses are shown in Column 3. Columns 4-6 are concentration determinations based on binding to D3 monoclonal antibody immobilized in a BIAcore™ flow cell (an assay for TF domain structural/functional integrity), trypsin inhibition (an assay for Kunitz domain structural/functional integrity), and absorbance at 280 nm (total protein), respectively, for representative preparations of each fusion protein. ND = not determined.

### Example 3

#### Fusion proteins inhibit TF-FVIIa-dependent factor X activation

- The relative potency of the fusion proteins for inhibiting the catalytic function of the membrane tissue factor (mTF)-Factor VIIa (mTF-FVIIa) complex was evaluated by using an assay of FX activation. In this assay, FX is added to a solution of mTF-FVIIa and the rate of FXa formation is determined by removing aliquots at various times, quenching the reaction by addition of EDTA to chelate calcium, and then measuring the amount of FXa formed by using the FXa specific substrate SPECTROZYME™ FXa. FXa cleavage of SPECTROZYME™ FXa does not require calcium. Hydrolysis of SPECTROZYME™ FXa is monitored by absorbance measurements at 405 nM. The rate may be used to calculate the FXa concentration by reference to a standard curve constructed with purified FXa. FX activation assays were conducted in a microtiter format and absorbance changes were monitored on an SLT EAR340AT plate reader controlled by a Macintosh IICI computer equipped with DeltaSoftII software (Biometallics). Nonlinear regression analysis was carried out using KaleidaGraph v3.01 (Synergy Software).

noncovalent mixture of TF7I-C and hTFAA were slightly more potent than TF7I-C alone in inhibiting the intrinsic pathway of blood coagulation. An inhibitor concentration of about 200 nM for either KDTF5 or the TF7I-C/hTFAA mixture gave a 2-fold prolongation in clotting time. A TF7I-C concentration of 400 nM was required to give a 2-fold prolongation in clotting time. At the concentrations tested, hTFAA alone had no effect on the clotting time determined in an APTT assay.

#### Example 6

##### Determination of antithrombotic potential in a rabbit model of deep medial injury

Male New Zealand white rabbits (~4 kg) are anesthetized to surgical anesthesia plane with an IM injection of Ketamine / Xylazine. The rabbits are placed supine on a restraining board, warmed to 37 °C, and the neck and inner thigh area shaved. Teflon catheters are placed in a marginal ear vein and femoral artery for drug delivery and sample collection respectively. Prior to treatment, blood samples are collected for coagulation tests (APTT and PT). Bleeding time is assessed from a cut made in the cuticle portion of a hind limb nail. Incisions are made in the neck region and the entire left common carotid artery and its branches are surgically isolated. An ultrasonic flow probe (Transonics®) is placed on the common carotid approximately 5 cm caudal to the common - internal bifurcation. After blood flow reaches a stable baseline, drugs (saline or test compounds) are delivered via the marginal ear vein. A deflated embolectomy catheter (Fogarty®, 3F) was then introduced into the lumen of the common carotid via an incision in the lingual branch. Blood flow through the artery is stopped briefly while the catheter is introduced and loosely secured with 2-0 silk tie at the incision site. After the catheter is in place and secure, blood flow is restored. The deflated balloon is advanced to within 2 mm of the flow probe and inflated with saline until resistance of the vessel wall is felt. The catheter is pulled back with a steady motion to the first branch and then deflated.

This procedure is repeated several times for each experimental animal, after which the catheter is removed. The ballooning procedure, from first insertion to removal of the catheter takes approximately 3 to 5 minutes and results in an area of damage that is 1.5 to 2 cm in length. Over 40 minutes, blood samples are taken for PT measurements, cuticle bleeding times are assessed and blood flow through the carotid monitored. Duration of patency is defined as the total amount of time (maximum = 40 minutes) that any measurable blood flow is detected in the artery. Patency rate refers to the percentage of animals tested who had carotid artery blood flow  $\geq$  5 minutes.

At the end of the experiment, the rabbit is euthanized and the carotid artery removed and opened. If any thrombus is present, it is removed, blotted and the weight recorded.

of FX activation for each inhibitor concentration. These values are plotted in Figure 6. Nonlinear regression analysis by using equation 1 provided the  $K_i^*$  values of  $160 \pm 19$  nM,  $3.8 \pm 0.5$  nM, and  $2 \pm 6$  pM for hTFAA, TF7I-C, and KDTF5, respectively. It is clear that fusion of hTFAA and TF7I-C with a linker having an estimated length of 60 Å increases the inhibitor potency by at least 100-fold.

5 **Equation 1:**

$$V_i/V_o = 1 - \frac{[E_o] + [I_o] + K_i^* - \sqrt{([E_o] + [I_o] + K_i^*)^2 - (4 \cdot [E_o] \cdot [I_o])}}{2 \cdot [E_o]} \quad (1)$$

In this equation  $[E_o]$  is the enzyme concentration,  $[I_o]$  is the inhibitor concentration,  $V_i$  is the initial velocity of FXa generation in the presence of  $[I_o]$  and  $V_o$  is the initial velocity in the absence of inhibitor.

**Example 5**

10 **Coagulation Assays**

Clotting times were measured using the ACL 300 Research Coagulation Analyzer. For the prothrombin time (PT) assays, the incubation time was set at 120 sec and acquisition time at 600 sec. Membranes from 293 cells expressing full length TF (Paborsky, L. R. et al., (1989) *Biochemistry* 28:8072-8077) were premixed with 25 mM  $\text{CaCl}_2$ . Citrated normal human plasma and inhibitor were incubated together for at least 5 minutes prior to assay. The sample (plasma and inhibitor) and reagent ( $\text{CaCl}_2/\text{TF}$ ) were automatically mixed together after a 2 min incubation at 37 °C. The clotting time was determined by optical assessment. Final concentrations were 0.028 to 5.6 µM inhibitor, 0.5 nM mTF, 12.5 mM  $\text{CaCl}_2$ , and 50% plasma in a total volume of 160 µL.

For the activated partial thromboplastin time (APTT) assays, the activation time was set at 70 sec and acquisition time at 360 sec. Citrated normal human plasma and inhibitor were incubated together for at least 5 minutes prior to assay. The sample (plasma and inhibitor) and activator (Instrumentation Laboratories Cephalin + Activator APTT Reagent) were automatically pipetted and incubated together for 70 sec at 37 °C, then  $\text{CaCl}_2$  was added and clotting time determined by means of optical assessment. Final concentrations were 0.028 to 5.6 µM inhibitor, 10 % activator, 10.0 mM  $\text{CaCl}_2$ , and 50.0 % plasma in a total volume of 200 µL.

**Results-**

25 The fusion protein KDTF5 gave a very potent inhibition of clotting in the PT assay as shown in Figure 7. KDTF5 was more potent than TF7I-C or hTFAA alone and also inhibited at a lower dose than observed for a mixture of TF7I-C and hTFAA. With KDTF5 a 2-fold prolongation in clotting time was obtained at an inhibitor concentration of about 800 nM. A 2-fold prolongation in clotting time was obtained at 1.9 µM for the equimolar mixture of TF7I-C and hTFAA. A concentration of 4 µM hTFAA alone gave a 2-fold increase in clotting time whereas a 2-fold prolongation was not obtained with TF7I-C at the highest concentration tested. These data show that KDTF5 is a potent inhibitor of TF dependent clotting and has greater potency than the noncovalent mixture of the two inhibitors.

As shown in Figure 8, KDTF5 retained the effect on clotting time in an APTT assay previously observed for TF7I-C alone (Dennis, M. S. & Lazarus, R. A. *J. Biol. Chem.* 269: 22129-22136 [1994]). Both KDTF5 and the

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
- (ii) TITLE OF INVENTION: Novel Factor VIIa Inhibitors
- 5 (iii) NUMBER OF SEQUENCES: 57
- (iv) CORRESPONDENCE ADDRESS:
- 10 (A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 460 Point San Bruno Blvd  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
- 15 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
- 20 (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- 25 (A) NAME: Kubinec, Jeffrey S.  
(B) REGISTRATION NUMBER: 36,575  
(C) REFERENCE/DOCKET NUMBER: P0958BPCT
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 415/225-8228  
(B) TELEFAX: 415/952-9881  
(C) TELEX: 910/371-7168

## 30 (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:2:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

45 Met His Ser Phe Cys Ala Phe Lys Ala Asp  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp  
1 5 10
- (2) INFORMATION FOR SEQ ID NO:4:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp  
15 1 5 10
- (2) INFORMATION FOR SEQ ID NO:5:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
Lys Glu Asp Ser Cys Gln Leu Gly Tyr Ser  
1 5 10
- (2) INFORMATION FOR SEQ ID NO:6:
- 25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:  
30 Thr Val Ala Ala Cys Asn Leu Pro Ile Val  
1 5 10
- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
Leu Pro Asn Val Cys Ala Phe Pro Met Glu  
1 5 10
- 40 (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 10 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear
- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 10 amino acids  
    (B) TYPE: Amino Acid  
    (D) TOPOLOGY: Linear
- 5     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp  
    1                    5                    10
- (2) INFORMATION FOR SEQ ID NO:4:
- 10     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 10 amino acids  
        (B) TYPE: Amino Acid  
        (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
Gly Pro Ser Trp Cys Leu Thr Pro Ala Asp  
15     1                    5                    10
- (2) INFORMATION FOR SEQ ID NO:5:
- 20     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 10 amino acids  
        (B) TYPE: Amino Acid  
        (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:  
Lys Glu Asp Ser Cys Gln Leu Gly Tyr Ser  
          1                    5                    10
- (2) INFORMATION FOR SEQ ID NO:6:
- 25     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 10 amino acids  
        (B) TYPE: Amino Acid  
        (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:  
30     Thr Val Ala Ala Cys Asn Leu Pro Ile Val  
          1                    5                    10
- (2) INFORMATION FOR SEQ ID NO:7:
- 35     (i) SEQUENCE CHARACTERISTICS:  
        (A) LENGTH: 10 amino acids  
        (B) TYPE: Amino Acid  
        (D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
Leu Pro Asn Val Cys Ala Phe Pro Met Glu  
          1                    5                    10
- 40     (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:  
            (A) LENGTH: 10 amino acids  
            (B) TYPE: Amino Acid  
            (D) TOPOLOGY: Linear
- 45     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

WO 97/20939

Arg Pro Asp Phe Cys Leu Glu Pro Pro Tyr  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe  
 1 5 10 14

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe  
 1 5 10 14

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe  
 1 5 10 14

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Phe Tyr Tyr Asn Ser Val Ile Gly Lys Cys Arg Pro Phe  
 1 5 10 14

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Tyr Phe Tyr Asn Gly Thr Ser Met Ala Cys Glu Thr Phe  
 1 5 10 14

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

5 Leu Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe  
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:15:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Trp Tyr Tyr Asp Pro Asn Thr Lys Ser Cys Ala Arg Phe  
1 5 10 14

15 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Trp Phe Phe Asn Phe Glu Thr Gly Glu Cys Glu Leu Phe  
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:17:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

30 Arg Tyr Phe Tyr Asn Ala Lys Ala Gly Leu Cys Gln Thr Phe  
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:18:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Ala Ala Val  
1 5 10 15

40 Cys Gly Ser Ala  
19

(2) INFORMATION FOR SEQ ID NO:19:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear



WO 97/20939

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val  
 1 5 10 15

Cys Gly Ser Ala  
 19

5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Asn Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met  
 1 5 10 15

Cys Thr Arg Asp  
 19

15

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile  
 1 5 10 15

Cys Glu Asp Gly  
 19

25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala  
 1 5 10 15

Cys Lys Lys Gly  
 19

35

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Asn Gly Asn Asn Phe Val Thr Glu Lys Glu Cys Leu Gln Thr  
 1 5 10 15

Cys Arg Thr Val

19

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr  
1 5 10 15  
10 Cys Gly Val Pro  
19

## (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gly Asn Glu Asn Lys Phe Gly Ser Gln Lys Glu Cys Glu Lys Val  
1 5 10 15  
20 Cys Ala Pro Val  
19

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Gly Asn Ser Asn Asn Phe Leu Arg Lys Glu Lys Cys Glu Lys Phe  
1 5 10 15  
30 Cys Lys Phe Thr  
19

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 19 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ala Lys Arg Asn Asn Phe Lys Ser Ala Glu Asp Cys Met Arg Thr  
1 5 10 15  
40 Cys Gly Gly Ala  
19

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids

(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

5 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Val Cys Arg  
1 5 10 15  
Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe  
35 40 45  
10 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID-NO:29:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Trp Cys Arg  
1 5 10 15  
20 Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe  
35 40 45  
25 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:  
30 (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Phe Cys Arg  
1 5 10 15  
35 Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

40 (2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Gly Gly Trp Cys Arg
 1              5              10              15
Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys
5              20              25              30
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe
              35              40              45
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala
              50              55              58

```

10 (2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg
 1              5              10              15
Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys
              20              25              30
20 Ala Pro Phe Phe Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe
              35              40              45
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala
              50              55              58

```

(2) INFORMATION FOR SEQ ID NO:33:

25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```

30 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Trp Cys Arg
    1              5              10              15
Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys
              20              25              30
Ala Pro Phe Ile Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe
35              35              40              45
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala
              50              55              58

```

(2) INFORMATION FOR SEQ ID NO:34:

40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys

```

	1		5		10		15
	Ala	Met	Ile	Ser	Arg	Trp	Tyr
					20	Phe	Asp
						25	Val
							Thr
							Glu
							Gly
							Lys
							Cys
							30

5	Ala	Pro	Phe	Ile	Tyr	Gly	Gly
							Cys
							35
							Trp
							Gly
							40
							Asn
							Arg
							Asn
							Asn
							Phe
							45

	Asp	Thr	Glu	Glu	Tyr	Cys	Ala
							Ala
							50
							Val
							Cys
							55
							Gly
							Ser
							Ala
							58

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
- 10 (A) LENGTH: 58 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

15	Val	Arg	Glu	Val	Cys	Ser	Glu
	1				5		Gln
							Ala
							10
							Glu
							Thr
							Gly
							Pro
							Cys
							Arg
							15

	Ala	Leu	Ile	Ser	Arg	Trp	Tyr
							Phe
							20
							Asp
							Val
							25
							Thr
							Glu
							Gly
							Lys
							Cys
							30

	Ala	Pro	Phe	Trp	Tyr	Gly	Gly
							Cys
							35
							Gly
							Gly
							40
							Asn
							Arg
							Asn
							Asn
							Phe
							45

20	Asp	Thr	Glu	Glu	Tyr	Cys	Ala
							Ala
							50
							Val
							Cys
							55
							Gly
							Ser
							Ala
							58

## (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 58 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

	Val	Arg	Glu	Val	Cys	Ser	Glu
	1				5		Gln
							Ala
							10
							Glu
							Thr
							Gly
							Pro
							Cys
							Arg
							15

30	Ala	Leu	Ile	Ser	Arg	Trp	Tyr
							Phe
							20
							Asp
							Val
							25
							Thr
							Glu
							Gly
							Lys
							Cys
							30

	Ala	Pro	Phe	Tyr	Tyr	Gly	Gly
							Cys
							35
							Gly
							Gly
							40
							Asn
							Arg
							Asn
							Asn
							Phe
							45

35	Asp	Thr	Glu	Glu	Tyr	Cys	Ala
							Ala
							50
							Val
							Cys
							55
							Gly
							Ser
							Ala
							58

## (2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
- 40 (A) LENGTH: 58 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

	Val	Arg	Glu	Val	Cys	Ser	Glu
	1				5		Gln
							Ala
							10
							Pro
							Gly
							Val
							Cys
							Arg
							15

	Ala	Leu	Ile	Leu	Arg	Trp	Tyr
							Phe
							Asp
							Val
							Thr
							Glu
							Gly
							Lys
							Cys

				20						25					30
	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Tyr	Gly	Asn	Arg	Asn	Asn	Phe
					35					40					45
5	Asp	Thr	Glu	Glu	Tyr	Cys	Ala	Ala	Val	Cys	Gly	Ser	Ala		
					50					55			58		

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

15 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Leu Cys Arg  
1 5 10 15  
Ala Leu Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Phe Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

20 (2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	Ala	Glu	Pro	Gly	Trp	Cys	Arg
	1				5					10					15
	Ala	Leu	Ile	Leu	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys
					20					25					30
30	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Tyr	Gly	Asn	Arg	Asn	Asn	Phe
					35					40					45
	Asp	Thr	Glu	Glu	Tyr	Cys	Ala	Ala	Val	Cys	Gly	Ser	Ala		
					50					55			58		

(2) INFORMATION FOR SEQ ID NO:40:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

40	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	Ala	Glu	Pro	Gly	Pro	Cys	Arg
	1				5					10					15
	Ala	Leu	Ile	Leu	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys
					20					25					30
	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe

35

40

45

Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## (2) INFORMATION FOR SEQ ID NO:41:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

10 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15  
Ala Met Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
15 Ala Pro Phe Ile Tyr Gly Gly Cys His Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## (2) INFORMATION FOR SEQ ID NO:42:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

25 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15  
Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Val Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
35 40 45  
30 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## (2) INFORMATION FOR SEQ ID NO:43:

- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15  
40 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Val Tyr Gly Gly Cys Phe Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala

50

55

58

## (2) INFORMATION FOR SEQ ID NO:44:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15  
10 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Ile Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
35 40 45  
15 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## (2) INFORMATION FOR SEQ ID NO:45:

## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15  
25 Ala Met Tyr Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
Ala Pro Phe Ile Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## 30 (2) INFORMATION FOR SEQ ID NO:46:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

## 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Val Cys Arg  
1 5 10 15  
Ala Met Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30  
40 Ala Pro Phe Ile Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe  
35 40 45  
Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

## (2) INFORMATION FOR SEQ ID NO:47:



- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15

Ala Leu Met Arg Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30

10 Ala Pro Phe Tyr Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
 35 40 45

Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

(2) INFORMATION FOR SEQ ID NO:48:

- 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

20 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15

Ala Ile Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30

25 Ala Pro Phe Ile Tyr Gly Gly Cys His Gly Asn Arg Asn Asn Phe  
 35 40 45

Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

(2) INFORMATION FOR SEQ ID NO:49:

- 30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

35 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15

Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30

Ala Pro Phe Tyr Tyr Gly Gly Cys His Gly Asn Arg Asn Asn Phe  
 35 40 45

40 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

(2) INFORMATION FOR SEQ ID NO:50:

- 45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15  
 5 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30  
 Ala Pro Phe Trp Tyr Gly Gly Cys Trp Gly Asn Arg Asn Asn Phe  
 35 40 45  
 10 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 15 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15  
 20 Ala Met Ile Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30  
 Ala Pro Phe Leu Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
 35 40 45  
 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

25 (2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys  
 1 5 10 15  
 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
 20 25 30  
 35 Ala Pro Phe Phe Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
 35 40 45  
 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
 50 55 58

(2) INFORMATION FOR SEQ ID NO:53:

40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

WO 97/20939

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys 15  
 1 5 10  
 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys 30  
 20 25  
 5 Ala Pro Phe Tyr Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe 45  
 35 40  
 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala 58  
 50 55

(2) INFORMATION FOR SEQ ID NO:54:

- 10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

15 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Lys 15  
 1 5 10  
 Ala Leu Met Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys 30  
 20 25  
 20 Ala Pro Phe Val Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe 45  
 35 40  
 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala 58  
 50 55

(2) INFORMATION FOR SEQ ID NO:55:

- 25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

30 Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Ala Cys Lys 15  
 1 5 10  
 Ala Met Tyr Lys Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys 30  
 20 25  
 Ala Pro Phe Ile Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe 45  
 35 35 40  
 35 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala 58  
 50 55

(2) INFORMATION FOR SEQ ID NO:56:

- 40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 58 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Gly Arg 15  
 1 5 10

Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30

Ala Pro Phe Phe Tyr Gly Gly Ala Tyr Gly Asn Arg Asn Asn Phe  
35 40 45

S Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
                  50                      55                      58

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 58 amino acids  
(B) TYPE: Amino Acid  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Val Arg Glu Val Cys Ser Glu Gln Ala Glu Pro Gly Pro Cys Arg  
1 5 10 15

15 Ala Leu Ile Leu Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys  
20 25 30

Ala Pro Phe Phe Tyr Gly Gly Cys Tyr Gly Asn Arg Asn Asn Phe  
35 40 45

20 Asp Thr Glu Glu Tyr Cys Ala Ala Val Cys Gly Ser Ala  
50 55 58

WO 97/20939

## WHAT IS CLAIMED IS:

1. A fusion protein comprising:
  - a) a Factor VIIa ("FVIIa") active site inhibitor domain;
  - b) a linker domain; and
  - c) a tissue factor ("TF") domain.
2. The fusion protein according to claim 1 wherein said FVIIa active site inhibitor domain is a reversible serine protease inhibitor.
3. The fusion protein according to claim 1 wherein said FVIIa active site inhibitor domain is an irreversible serine protease inhibitor.
4. The fusion protein according to claim 2 wherein said reversible serine protease inhibitor comprises a Kunitz domain ("KD").
5. The fusion protein of claim 4 wherein said linker domain comprises a  $-(\text{Gly})_4\text{-Ser-}$  linker module.
6. The fusion protein of claim 5 wherein said linker domain comprises four  $-(\text{Gly})_4\text{-Ser-}$  linker modules.
7. The fusion protein of claim 5 wherein said linker domain comprises five  $-(\text{Gly})_4\text{-Ser-}$  linker modules.
8. The fusion protein of claim 3 having the formula:  

$$\text{KD}-[(\text{Gly})_4\text{-Ser}]_x\text{-TF}$$
 wherein KD is a Kunitz type active site inhibitor of Factor VIIa;  
 X is an integer between 1 and 10; and  
 TF is tissue factor domain.
9. The fusion protein of claim 8 wherein the tissue factor variant is hTFAA.
10. The fusion protein of claim 9 wherein the KD is TF71-C.
11. The fusion protein of claim 10 wherein x is 4.
12. The fusion protein of claim 10 wherein x is 5.
13. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and the fusion protein of claim 1.
14. A method for inhibiting human Factor VIIa (FVIIa) procoagulant activity comprising contacting said FVIIa with the fusion protein of claim 1 in an amount sufficient to inhibit the procoagulant activity of said TF-FVIIa.
15. A method for inhibiting human tissue factor-Factor VIIa (TF-FVIIa) procoagulant activity in a mammal comprising administering a therapeutically effective amount of the composition of claim 13 to the mammal.
16. The method of Claim 15 further comprising administering the composition in combination with a thrombolytic agent.
17. The method of Claim 15 further comprising administering the composition in combination with an anticoagulant.

18. A method of treating a mammal for which inhibiting Factor VIIa is indicated comprising administering a pharmaceutically effective amount of the composition of Claim 13 to the mammal.
19. An isolated DNA molecule encoding the fusion protein of Claim 8.
20. The DNA molecule of claim 19 further comprising an expression control sequence operably  
5 linked to the DNA molecule.
21. An expression vector comprising the DNA molecule of claim 20 wherein the control sequence is recognized by a host cell transformed with the vector.
22. The vector of claim 21 that is a plasmid.
23. A host cell transformed with the plasmid of Claim 22.
- 10 24. A method for expressing a DNA molecule encoding a serine protease inhibitor in a host cell, comprising culturing the host cell of claim 23 under conditions suitable for expression of the inhibitor.
25. The method of claim 24 further comprising recovering the inhibitor from the culture medium.

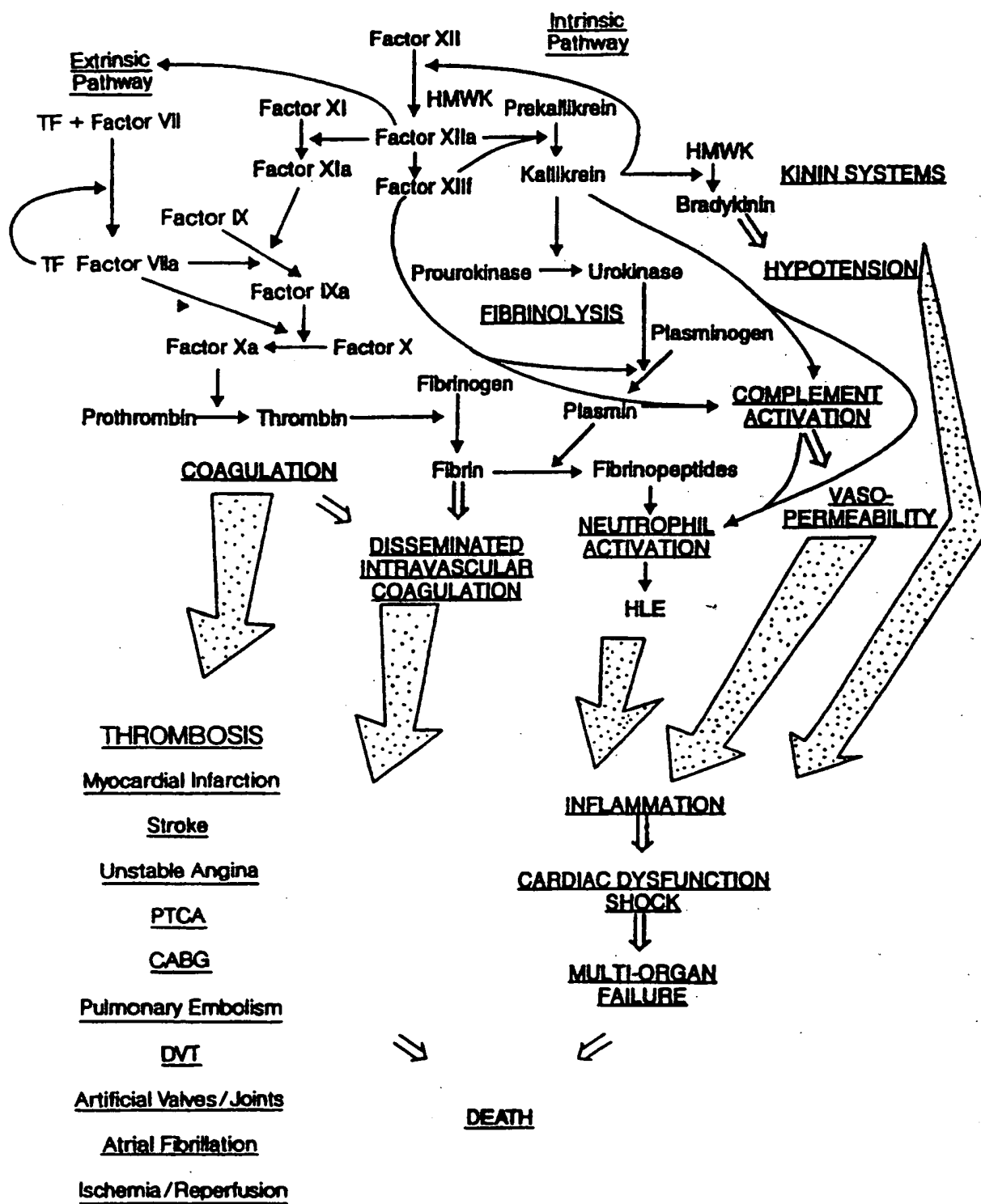


FIG. 1

2 / 8

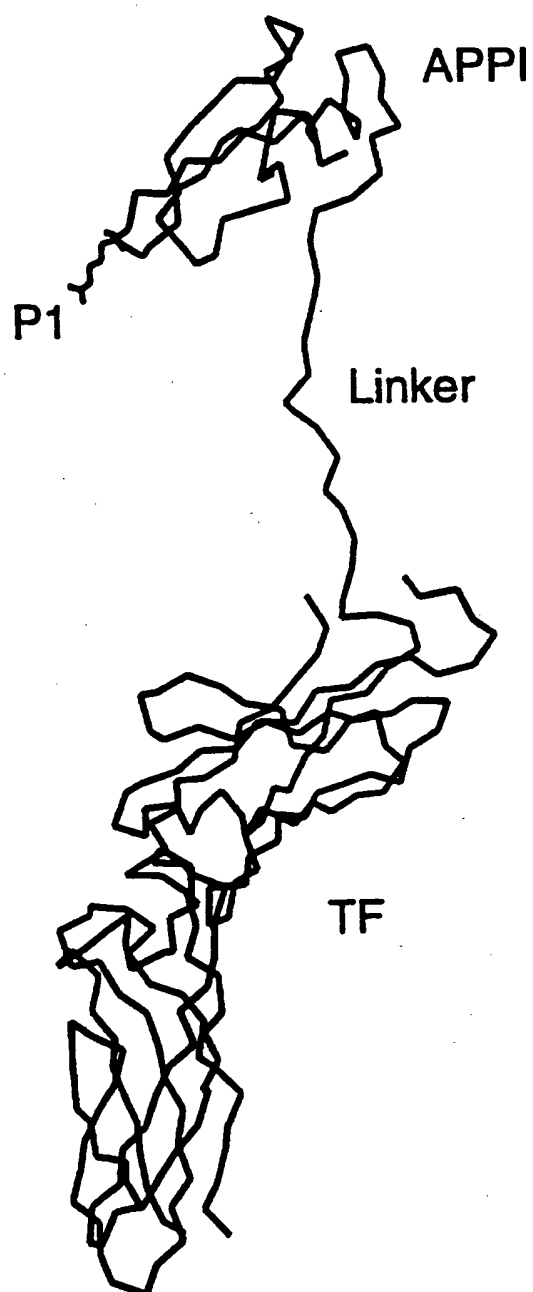


FIG. 2



3 / 8

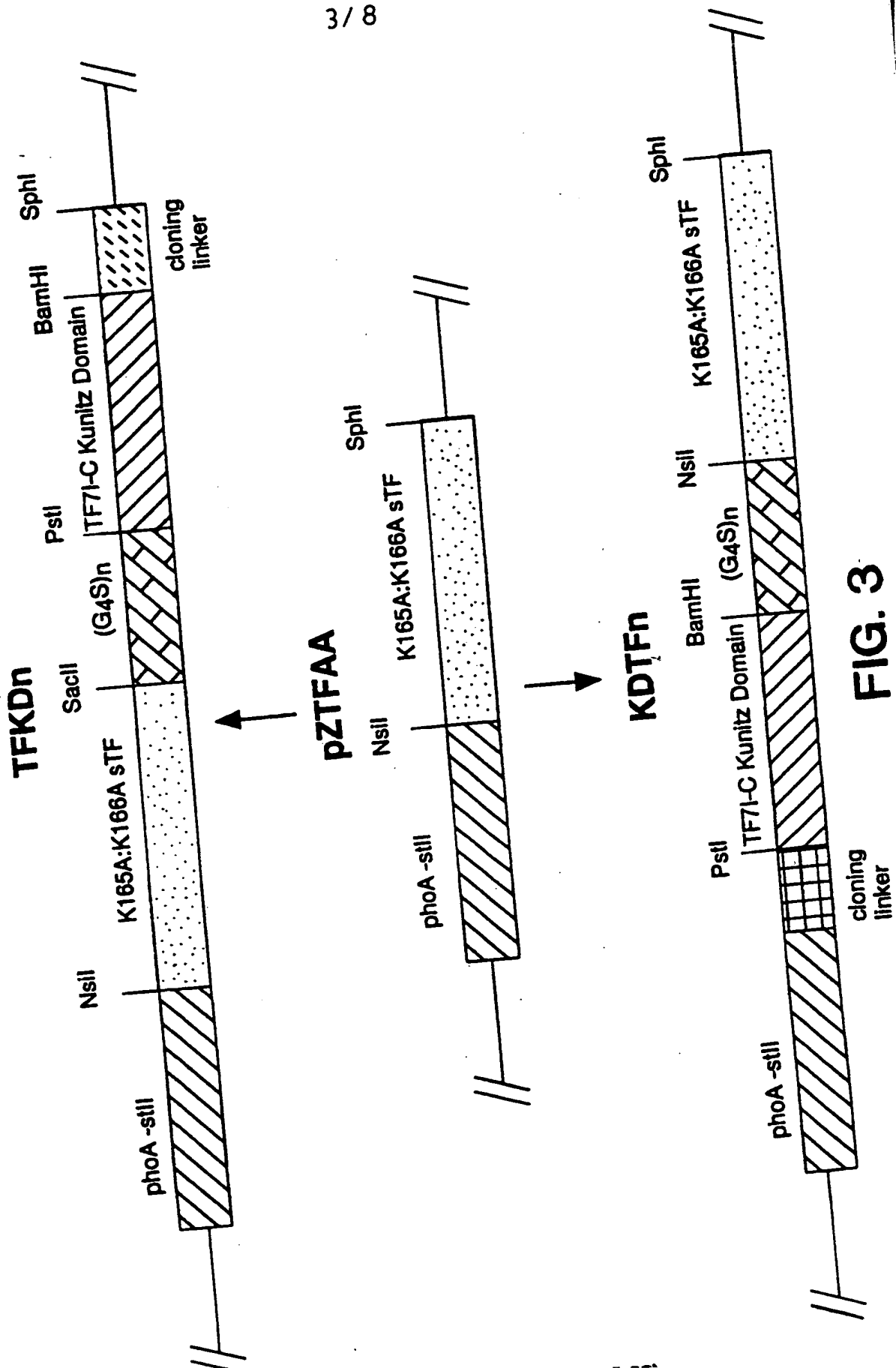


FIG. 3

4 / 8

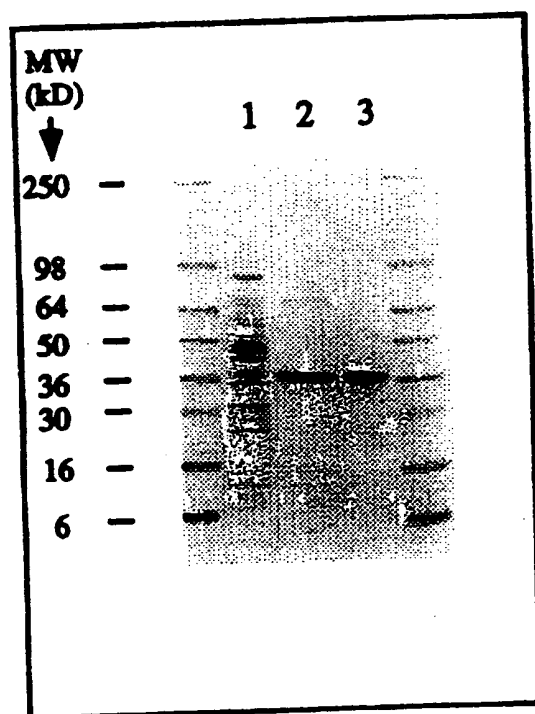


FIG. 4

5 / 8

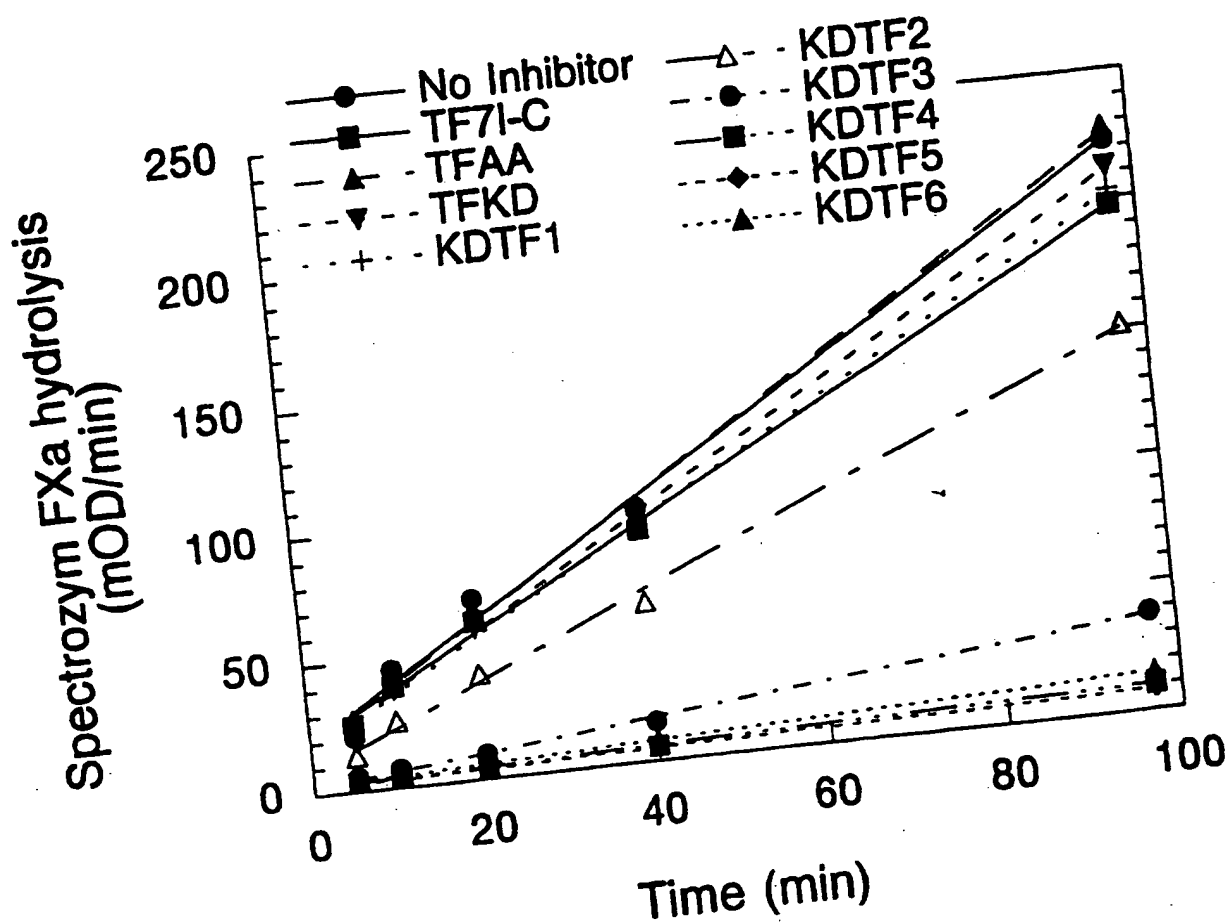


FIG. 5

6 / 8

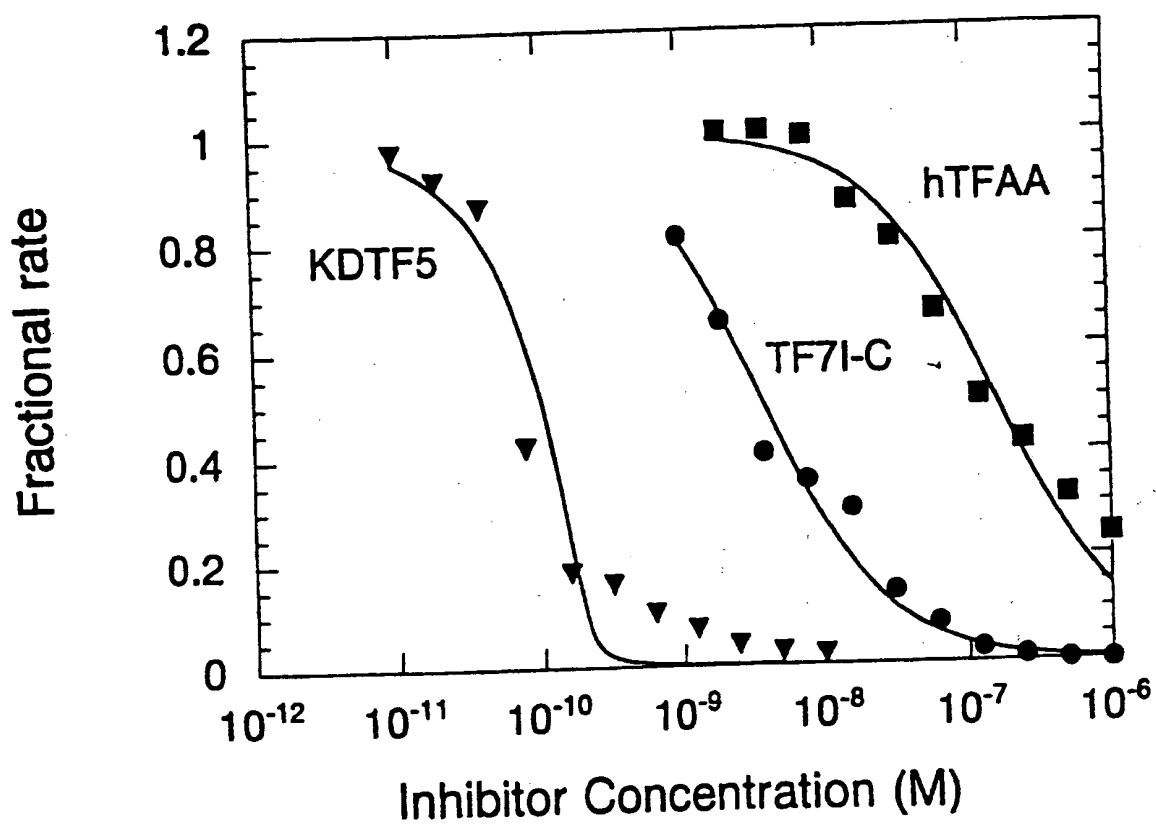


FIG. 6

7 / 8

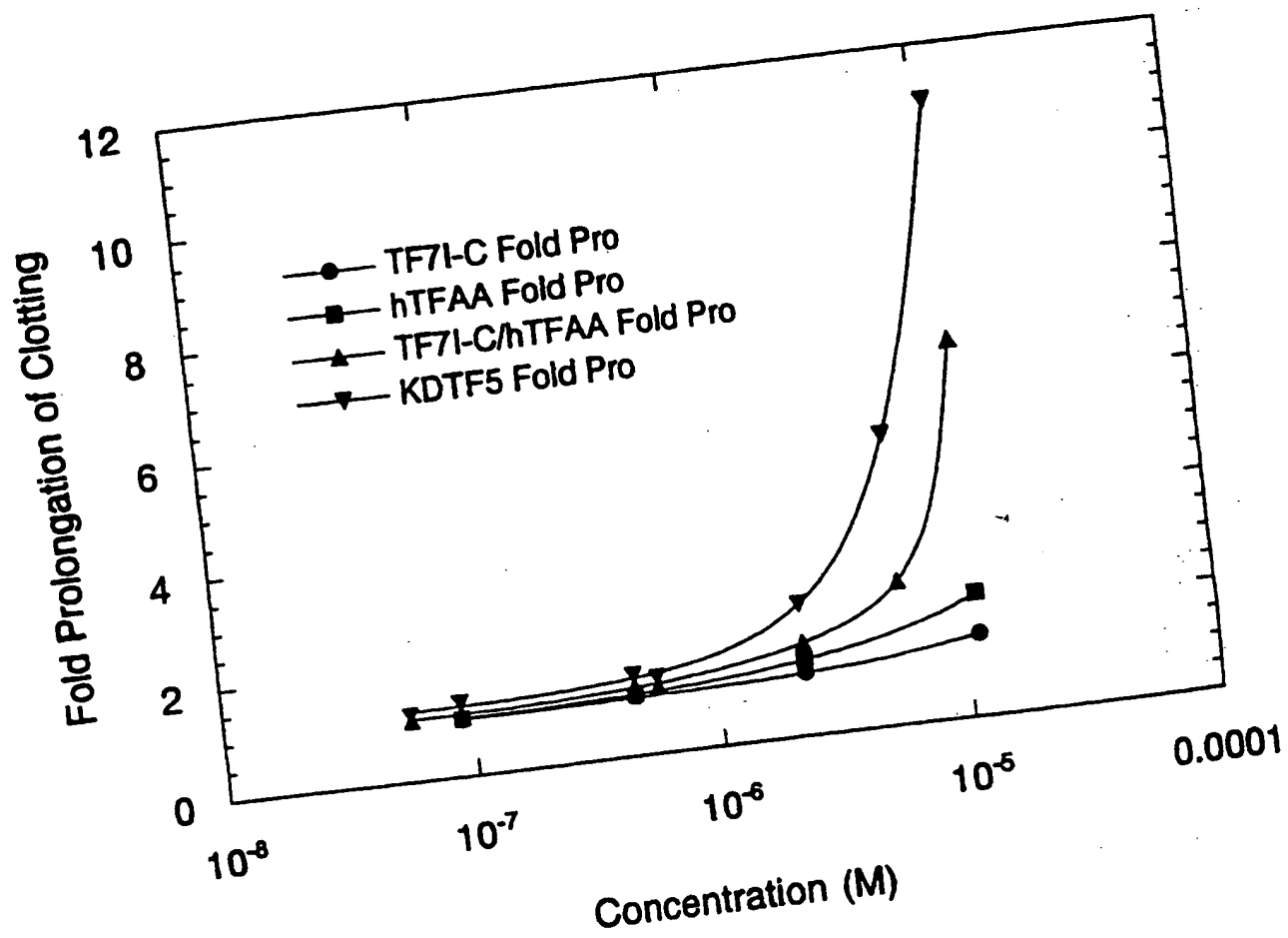


FIG. 7

8 / 8

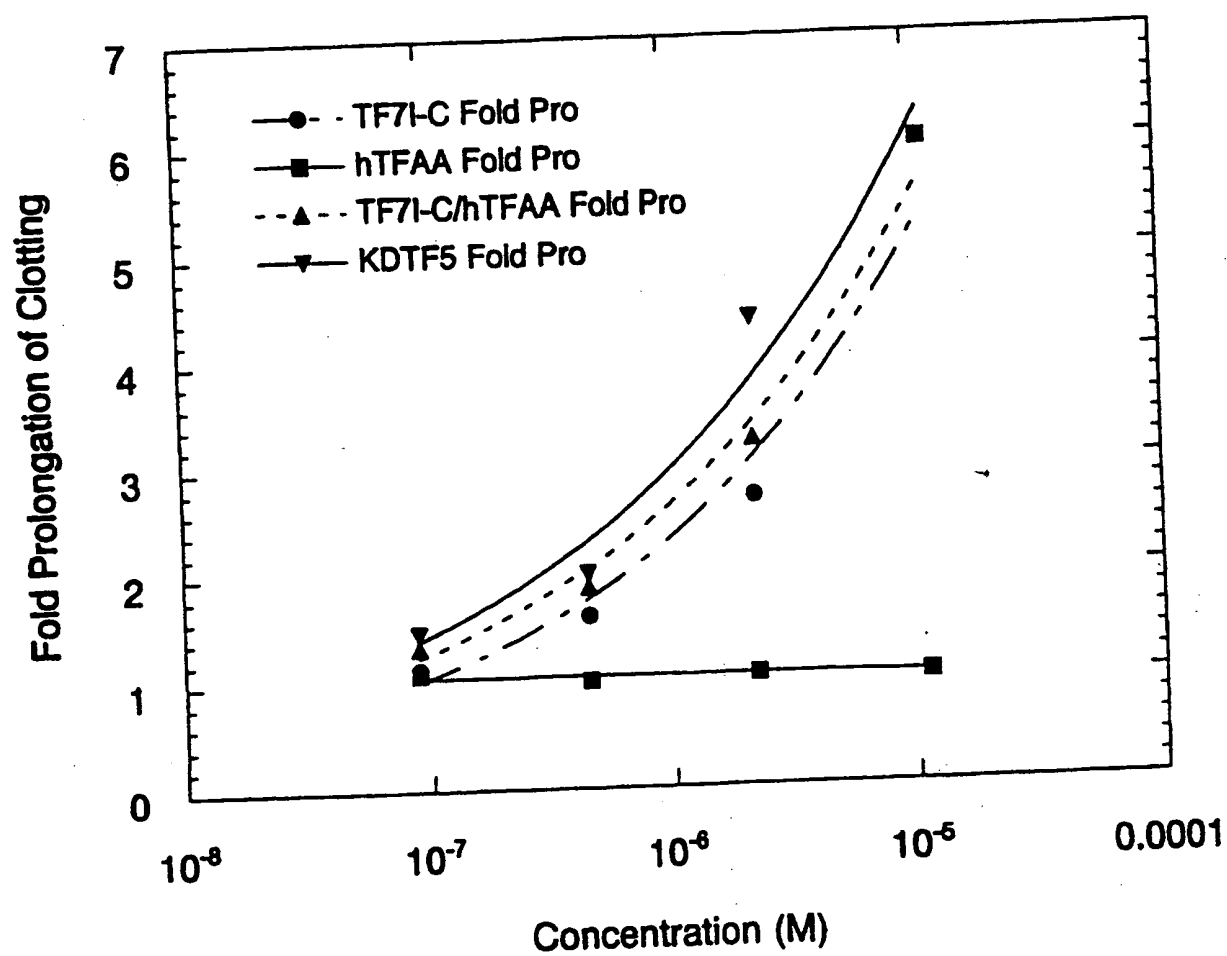


FIG. 8

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/18756

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C07K19/00 A61K38/36 A61K38/57 C12N1/21  
 //C07K14/745, C07K14/81

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 346 991 A (ROY SOUMITRA ET AL) 13 September 1994 cited in the application see the whole document ---	1-25
Y	WO 94 28017 A (SCRIPPS RESEARCH INST ; RUF WOLFRAM (US); EDGINGTON THOMAS S (US)) 8 December 1994 see the whole document ---	1-25
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 35, 2 September 1994, MD US, pages 222129-22136, XP002027734 M. DENNIS AND A. LAZARUS: "Kunitz domain inhibitors of tissue factor-factor VIIa I" cited in the application see the whole document ---	1-25

-/--

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*G\* document member of the same patent family

Date of the actual completion of the international search

21 March 1997

Date of mailing of the international search report

02.04.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Van der Schaal, C

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/18756

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 23860 A (GENENTECH INC) 8 September 1995 cited in the application see the whole document ---	1-25
Y	BLOOD, vol. 86, no. 10 suppl, 15 November 1995, page 91a XP002028014 KIRCHHOFFER D. ET AL: "Anticoagulant activity of different tissue factor/factor VIIa inhibitors in a human ex-vivo thrombosis model" see abstract 350.5 ---	1-25
Y	SCIENCE, vol. 248, no. 4961, 15 June 1990, pages 1421-1424, XP000253643 GIRARD T J ET AL: "INHIBITION OF FACTOR VIIA-TISSUE FACTOR COAGULATION ACTIVITY BY A HYBRID PROTEIN" see the whole document ---	1-25
Y	EP 0 439 442 A (UNIV WASHINGTON) 31 July 1991 see the whole document -----	1-25



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 18756

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14, 15-18  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 14, partially, and 15-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 96/18756

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5346991 A	13-09-94	US 5589363 A	31-12-96
WO 9428017 A	08-12-94	AU 7050994 A	20-12-94
WO 9523860 A	08-09-95	CA 2184058 A	08-09-95
		EP 0748380 A	18-12-96
EP 0439442 A	31-07-91	AT 135044 T	15-03-96
		CA 2034881 A	26-07-91
		DE 69117545 D	11-04-96
		DE 69117545 T	31-10-96
		ES 2086521 T	01-07-96
		JP 2560150 B	04-12-96
		JP 4211000 A	03-08-92

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**